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Regulation of water access, storage, separation and release of drugs from the carbon nanotube functionalized by cytosine rich DNA fragments

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ABSTRACT

We found that carmustine can be stored in the carbon nanotube (CNT) interior for a long time due to hydrophobic interactions. The access of water to carmustine phase in the CNT interior can be controlled by the state of cytosine rich DNA fragments covalently bound to the CNT tips and to the presence of doxorubicin molecules intercalated within bundles of DNA fragments. More effective control of water access and subsequent decomposition of carmustine due to the contact with water was observed when some small amount of doxorubicin molecules cork the CNT ends. Our analysis shows that carmustine decomposition products naturally separate when decomposition occurs within the CNT. The alkylating agent, chloroethyl carbonium cation, spontaneously escapes from the CNT but the carbamylation agent, chloroethyl isocyanate, is still kept within the nanotube interior. The separation process and release of the alkylating agent needs uncorking the nanotube by doxorubicin molecules. The latter process is likely to occur spontaneously at acidic pH when intercalation of doxorubicin within the DNA fragments becomes ineffective. The features of the proposed molecular model, obtained from molecular dynamics simulations, can be beneficial in design of novel smart drugs carriers to a tumor microenvironment revealing the reduced extracellular pH.

1. Introduction

In our recent studies we found that functionalization of carbon nanotubes, CNT, by short cytosine rich fragments of telomeric DNA, iM, can produce systems with interesting properties. Both noncovalent [1] and covalent [2] functionalizations led to obtaining pH sensitive drug carriers which however required some kind of activation by the presence of doxorubicin, DOX, molecules. The adsorption of iM molecules on the surface of CNT was weak without the presence of DOX, and, the structure of covalently linked iM chains (folded or unfolded, Fig. 1 A, B) did not reveal ability to regulate the access to the CNT interior of other molecules without DOX molecules intercalated in those chains. Thus, the mentioned molecular architecture of the carrier had to be activated by the presence of doxorubicin and then it became truly functional carrier of DOX itself.

Carbon nanotubes have been quite widely studied as carriers of small molecule drugs due to their empty internal volume and large external surface which could be further functionalized in order to produce biocompatibility [3–9]. Various anticancer drugs were studied as potential cargoes for delivery by CNTs [10]. However, carbon nanotubes always need additional treatment in order to reduce their possible toxicity, [11,12] improve solubility [13] or just to add additional function like selectivity to target site [14] and sensitivity to some triggering factor for the release of cargo [15,16].

Among possible factors which can produce property of controllable release of drugs from the CNT also the telomeric DNA fragments, iM, were studied. The iM is composed of the sequence 5'$(CCCTAA)_n$3' repeated at least four times. Then it reveals ability to performing reversible folding and unfolding into i-motif spatial shape in response to pH change [17,18]. That property of iM sequences was used several times in literature in construction of pH sensitive carriers of drugs [19–24]. The most frequently used drug in those studies was doxorubicin, DOX, a very well-known anticancer drug belonging to anthracycline group. DOX strongly interacts with the DNA strands and intercalates the duplex and finally inhibits the replication process. Strong interaction (or intercalation) of DOX with the unfolded iM sequences (Fig. 1A) and loss of that property in the case of folded iM sequences into i-motif (Fig. 1B) was found as the mechanism of action of

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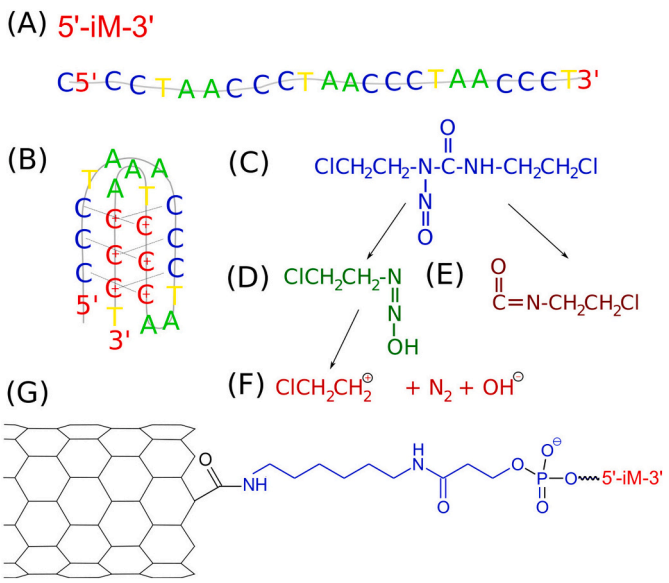

Fig. 1. (A) The unfolded cytosine rich fragment of telomeric DNA (5′-iM-3′) at the neutral pH, and (B) the same fragment after folding into i-motif at acidic pH. (C) is the chemical formula of carmustine, BCNU. (D) and (E) are its decomposition products. (F) is the true alkylating agents and it is formed due to decomposition of (D) in aqueous media. (G) is the schematic representation of the CNT-based drug carrier composed of 5′-iM-3′ chains linked to the CNT tips via the spacer molecule (blue fragment in (G)). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the drug carriers described in references [1,2]. In those models the role of doxorubicin was twofold: it was a kind of activating agent and, at the same time, it was the cargo encapsulated within the carrier. Thus, the functionalization of carbon nanotubes by iM molecules turned out to be very promising strategy for construction of pH sensitive carriers of DOX. Therefore it is useful to consider application of that molecular model and its mechanism of action in the case of the other drug molecules.

One of the interesting and useful molecules in chemotherapy is carmustine, (1,3-bis(2-chloroethyl)-1-nitrosourea, BCNU). This molecule has been used in treatment of glioblastoma which is a WHO grade IV brain tumor which represents one of the most lethal human cancers [25]. Carmustine belongs to the class of alkylating agents and induces interstrand crosslinks between the guanine and cytosine bases in DNA [26–28]. Carmustine is highly unstable in aqueous media and spontaneously decomposes into chloroethyl diazohydroxides (Fig. 1D) and chloroethyl isocyanate moieties (Fig. 1E) with half-life of a few minutes [27,28]. The latter is thought to be responsible for carbamylation of intracellular proteins while chloroethyl diazohydroxide decomposes further giving finally the true alkylating agent [26] which is the chloroethanyl ion (Fig. 1F). Thus, preventing carmustine from direct contact with water in intravenous administration and prior to contact with tumor tissue would be highly beneficial. Therefore a design of suitably tuned BCNU carrier which would be able to unload (or allow for its contact with water) the drug only at the target site would be an important achievement.

Combination of carbon nanotubes and BCNU in a single device has been addressed in two theoretical works [29,30]. In one of them carbon nanotube was capped by magnetic nanoparticles which were linked to the CNT tips by pH cleavable hydrazone linkers [29]. BCNU was encapsulated in the CNT interior and due to strong interaction of magnetic nanoparticles with CNT the permeation of water to the CNT interior was impossible. Only after application of external magnetic field the nanotube became uncapped and BCNU was allowed to release. In the second publication [30] the authors considered transport and interaction of BCNU encapsulated in the (5,5) CNT with an active site of glutathione reductase enzyme. Release of BCNU was considered as a whole molecule but the applied length of the nanotube was small so only a single BCNU molecule could be stored in its internal space. However, the motivation for that study was rather determination of the binding site within the protein and enzyme inhibition due to strong interaction with BCNU at that site.

The current study focuses on the analysis of possible application of iM functionalized carbon nanotubes as carriers of carmustine molecules and regulation of their contact with water molecules. The performed molecular dynamics studies of various molecular architectures of CNT-based systems led to several important conclusions. Namely, we found that application of doxorubicin as additional component may lead to construction of CNT-based pH controlled container of BCNU with regulation of water access by external factor which is the pH of solution. That container can also act as a ‘divider’ and ‘dispenser’ of BCNU decomposition products due to ability of selective releasing one of these products and stopping the other one product within the CNT internal space.

2. Methods

2.1. Definition of the analyzed systems

The cytosine rich telomeric DNA fragments, iM, were used as pH sensitive moieties in construction of the CNT-based drug carrier. All building blocks and step by step stages of molecular construction of the model of the carrier were partially described in details in our previous paper [2]. However, the most important stages and assumptions related to the model construction need some description. Fig. 1 shows the essential components of the system. The general architecture of the carrier is presented in Fig. 1G.

The atomic structure of (20,0) carbon nanotube (diameter 15 Å) was generated using self-designed script. The CNT tips were next assumed to be covalently functionalized by N-ethyl-N-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) molecules [31] by creating peptide bonds on one side and substituting of primary amine oligo DNA linkers on the second side. Technically, it was done by creating atomic coordinates pdb files for the whole molecule and using tleap program from the AmberTools16 package [32,33] in order to produce force field files for the whole construct. These force field files were next converted into gromacs format using the AcPyPe script [34] and are provided as the supplementary information. The application of the EDC molecule as the spacer molecule for linking DNA motifs with CNT has already been described in the literature [31].

Depending on pH value the DNA motifs may adopt either a random coil form Fig. 1A or fold into i-motif form Fig. 1B. The number of iM chains linked to the CNT, as shown in Fig. 1G, was four on each side of the CNT. This number was chosen mainly due to geometric/steric reasons. Larger number of iM chains would be difficult to fit within a relatively small area of (20,0) CNT tip.

We considered three different cases of incorporation of BCNU and DOX to the carrier structure. These cases were built and tested according to previously collected knowledge about the interaction of these molecules with carbon nanotubes and iM fragments. Additionally each case was considered in either neutral or acidic pH so finally we had to analyze 6 different systems. Note that in acidic pH the cytosines within the iM become partially protonated and it leads to folding of iM (Fig. 1A) into i-motif (Fig. 1B). Table 1 provides the essential information about each system.

The systems B-N and B-A were studied in order to identify how the changes in the spatial forms of iM affect the state of carmustine molecules encapsulated in the interior of the nanotube. Particularly the access of water to BCNU molecules inside the CNT was studied. In the systems BD-N and BD-A we incorporated DOX molecules to the area of iM chains and this approach was used to make the spatial structure of iM chains more responsive to pH changes. Finally, the systems BD2-N and

Table 1
Essential information about the analyzed systems. The applied CNT diameter and length were 15 Å and 100 Å, respectively. The water flux to the CNT was determined during simulation of every system.

System	Number of BCNU	Number of DOX	pH	Water flux, ns^{-1}
B-N	30	0	Neutral	218 ± 48
B-A	30	0	Acidic	197 ± 48
BD-N	30	20	Neutral	84 ± 30
BD-A	30	20	Acidic	160 ± 45
BD2-N	20	28	Neutral	16 ± 12
BD2-A	20	28	Acidic	54 ± 20

BD2-A were designed to better control the water access to BCNU phase within the nanotube. These two systems were constructed by addition of 8 DOX molecules directly to the CNT interior but close to their ends.

2.2. Force field and computational details

The applied force field for DNA molecules/motifs was amber ff99 with bsc1 modifications [35,36] while for the other species we used gaff branch of the amber force field [32,33]. Because application of gaff needs properly determined partial charges on atoms we applied for that purpose the RESP/ESP charge derive server [37,38]. The step by step instructions concerning the generation of force field sets of parameters and construction of simulation boxes can be found in our previous publication [2]. Technically, they were based on creation of atomic coordinates pdb files, submitting them to the RESP/ESP charge derive server, and using the resulting mol2 files in either tleap program or AcPyPe script for production of the force field topology files according to the general amber force filed (gaff) scheme [32–34]. That procedure was applied to CNT and its linkage with DNA motifs using EDC molecule, for doxorubicin, carmustine and all of its decomposition products. The mixing of bsc1 parameterization for DNA motifs and gaff for other components of the systems is fully justified since both branches of the amber force field are fully compatible.

The applied water model was TIP3P with particle mesh Ewald summation of long range electrostatic interactions. We also accounted for the presence of saline ions with the concentration of 0.145 M. The calculations were performed using gromacs [39] molecular dynamics engine in NPT ensemble with 2 fs integration timestep. The pressure and temperature were set to 310 K and 1 atm, respectively. The applied thermostat and barostat were Berendsen velocity rescaling and Parinello-Rahman, respectively, as implemented in gromacs molecular dynamics program [39].

2.3. Outline of the systems construction and research methodology

The applied methodology, various research approaches and concepts utilized in this study can be summarized as follows.

- The DOX, BCNU, EDC and BCNU decomposition products were first subjected to quantum chemical computations in order to determine the values of the partial charges. This was done using RESP/ESP charge derive server. The DNA fragments in bsc1 parameterization have all the components of the force field ready to use, including the values of the partial charges. The CNT was assumed to be charge neutral.
- The CNT was assumed to be on-tip functionalized by carboxyl groups and thus it was further functionalized by EDC molecule using its amino end. This step was done by applying the tleap program from AmberTools16 package. On each end of the CNT four EDC linkers were added in that way.
- The second ends of the EDC linkers were coupled with 5′ ends of C-rich DNA fragments using tleap program. Two cases were considered: the neutral C-rich fragment in the random coil form (Fig. 1A) and acidic C-rich fragment folded into i-motif (Fig. 1B). In that way the systems with suffixes -N and -A were obtained and they correspond to ‘neutral.top’ and ‘acidic.top’ force field files in the supplementary material.
- The BCNU molecules were inserted to the internal space of CNT using self-designed scripts and next solvation was done using the standard gromacs module. The DOX molecules were inserted at the vicinity of the CNT ends using the previously obtained atomic structures and inputs. The force field files are available in the supplementary material as ‘DOX.itp’ and ‘BCNU.itp’.
- The decomposition of the selected BCNU molecules inside the CNT was done by replacing atomic coordinates of those BCNU molecules by coordinates of the decomposition products and modification of the force field files. (The names of the force field files are: ‘OCNCH2CH2CL.itp’ and ‘CH2CH2Cl.itp’) That replacement was done using fully relaxed structures obtained from runs with the BCNU molecules intact.
- The analysis of the numbers of molecules staying or escaping the CNT was done using simulation trajectories processed by self-designed scripts in Tcl code and using the VMD program.

3. Results and discussion

As already mentioned carmustine is a hydrophobic molecule and its interaction with CNT is strong. Indeed the results obtained from the analysis of systems B-N and B-A confirm that expectation. Incorporation of BCNU molecules to the internal space of CNT led to formation of dense and impermeable to water clusters. Fig. 2 shows simulation snapshots obtained after 100 ns of simulation of the B-N and B-A systems.

As seen in Fig. 2 the spatial structure of iM chains will be different depending on the assumed pH of solution. In the neutral case we can see that iM molecules are in forms of random coils and they are freely flanking in the bulk solution without any tendency to aggregate or adhere to the CNT surface. Similar behavior was already observed in our previous studies: on tip attachment of iM chains to the CNT leads to dendrimer-like structures of iM chains [2]. In the current study this behavior is even more pronounced because we do not see any interaction of BCNU with iM chains which was a fundamental observation in ref. [2]. The system B-A, representative to acidic pH, leads to formation of more dense structures around CNT tips due to more compact spatial forms of iM molecules, which are i-motifs. In both cases the CNT tips are either totally open to water (or other species from the bulk) or only very slightly obscured by fragments of i-motifs. Thus, the general and straightforward conclusion is that folding/unfolding transitions of iM chains do not regulate capping/uncapping of the nanotube tips.

The BCNU molecules due to their hydrophobicity form dense clusters inside the CNT. However, if there is no total filling of the inner CNT space by the BCNU molecules then water readily goes inside and fills the available space. This is better seen in Fig. 3 where plots of water density as a function of axial position in the CNT are shown. We can see that BCNU phase is impermeable to water in the areas of total filling the nanotube aperture. At the same time the density of water close to CNT ends is large and these areas are places of close contact of water and BCNU. However, the BCNU molecules move in the CNT collectively as the whole phase. Therefore, the areas of mixed density profiles appear due to instantaneous moves of the BCNU phase to the right or left and then water fills that space immediately. The peak of water density at z = 10 Å observed for B-N system was generated by an initial incorporation of some water molecules to the CNT by ‘gmx solvate_box’ command. As we can see in Fig. 2 for B-N that bulb resides in the CNT during the whole 100 ns run. However, we still observed some small exchange of water between this bulb and bulk water.

An intense exchange of water occurs at the areas of CNT tips. The right panel of Fig. 3 shows quantitatively determined factor describing that phenomenon. This is the flux toward the CNT interior and flux outside the CNT. The fluxes were determined as the numbers of

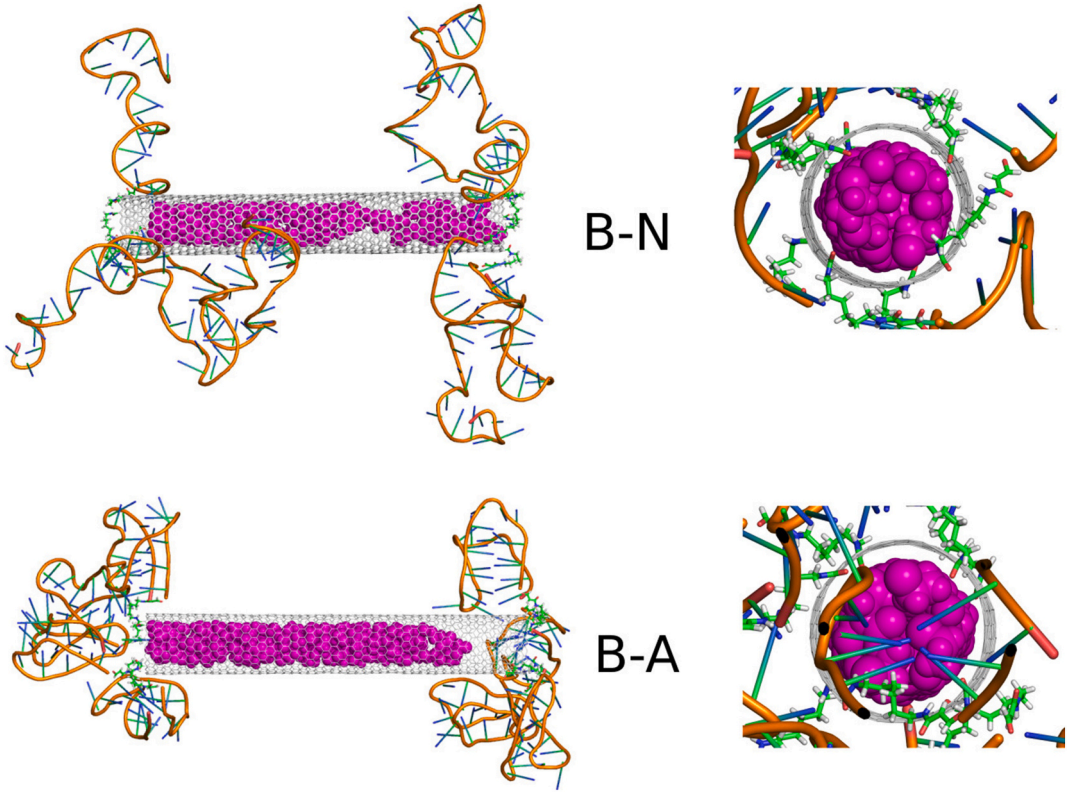

Fig. 2. Snapshots of the systems B-N and B-A after 100 ns simulations. The DNA fragments (iM) are drawn in cartoon representation while the magenta spheres show atoms of BCNU molecules. The right panels show edge views of CNT in order to underline that in both cases the CNT ends are open. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

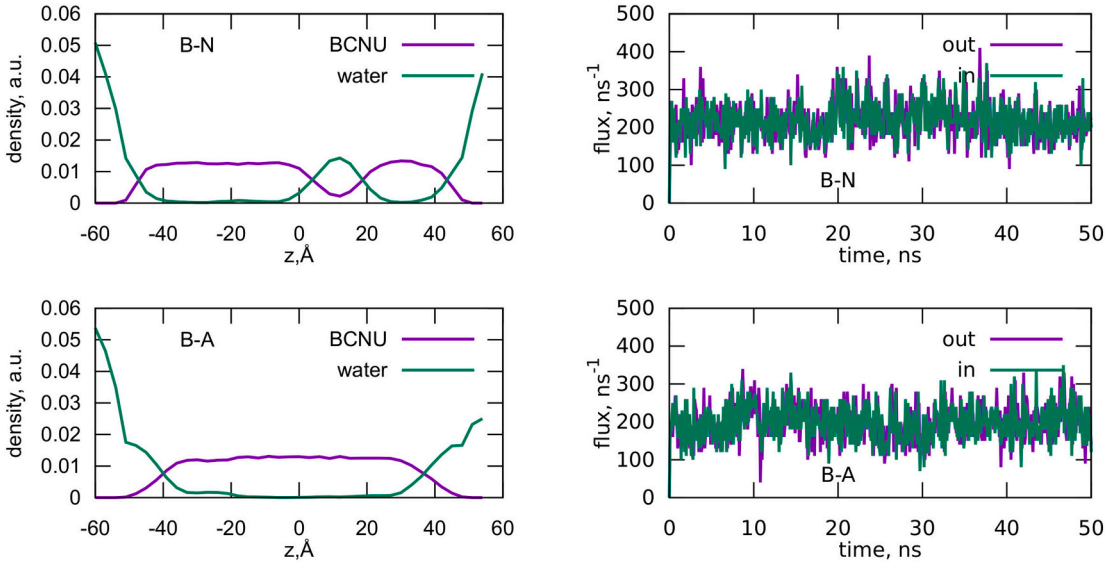

Fig. 3. Atomic density of water and BCNU molecules as a function of axial position within the CNT for both B-N and B-A systems (left panel). The net flux of water molecules into and out the nanotube (right panel) determined from the last 50 ns of simulations.

molecules which newly entered the CNT and just exited the CNT in given intervals of time. As seen in Fig. 3 these fluxes are actually constant in time and have similar values for both B-N and B-A systems. As shown in Table 1 the determined mean values of the fluxes are 218 ± 48 and 197 ± 48 molecules per nanosecond for the neutral and acidic pH, respectively. The conclusion is thus that the state of iM molecules (folded or unfolded) does not significantly affect the water flux into the CNT and its access to the BCNU phase. Moreover we also do not recognize any alteration of the iM structures or behavior due to the presence of BCNU in the CNT. In the other work [2] we found that doxorubicin slowly goes outside the CNT and next intercalate between iM chains at the neutral pH. Here, we do not observe a similar effect for the BCNU.

The observed in ref. [2] 'zipper' function of DOX suggested us application of DOX as the additional compound of carmustine carrier and led to construction and analysis of systems BD-N and BD-A. In these systems DOX molecules were initially put in the area of iM chains but

beyond the nanotube. The final structures obtained after long 100 ns simulation times are shown in Fig. 4.

The behavior of these systems is actually similar like the B-N and B-A: there is no escape of BCNU molecules from the nanotube either at neutral or in the acidic pH. In the BD-N system the BCNU phase split into two clusters, however, this is not very significant difference to the B-N system. More important and interesting is the influence of DOX molecules on the structure of iM chains, particularly at the neutral pH, BD-N. We can clearly see that the DNA chains have been clipped by the DOX molecules and formed clusters at the vicinity of the CNT ends. These clusters are however not able to cork the nanotube entrances but they effectively link all iM chains together. At acidic pH, BD-A, the DOX molecules are not able to link the i-motifs formed due to folding of iM fragments after protonation of cytosines. DOX is more mobile and readily diffuses on the CNT surface. We also found that at least one DOX molecule entered to CNT interior.

The density profiles shown in Fig. 5 also do not differ significantly from the corresponding profiles in Fig. 2. It is clearly seen that water has access to the BCNU phase no matter what is the state of iM chains crosslinked by DOX or not. The water density peak at $z \approx 0$ for the BD-N system indicates that the empty space in the corresponding Fig. 4 is filled in water.

The mean flux of water to the CNT (Table 1) is, however, significantly reduced when compared to the previous cases. Thus, the presence of DOX affects the access of water to the CNT interior because the fluxes in BD- systems are much smaller than in B- only systems. Moreover, we should underline that change of pH and the resulting change of iM chains structure and their interaction with DOX affects significantly the flux of water into the CNT. The flux at acidic pH is twice as that in the neutral pH thus the BCNU molecules are, at the neutral pH, 'protected' to some extent against interaction with water. Of course, this is not fully efficient protection because the flux is still significant.

The above effect suggested an another modification of the system structure which should reveal more pronounced regulation of water access to the BCNU phase localized inside the CNT. This is a system in which some parts of the CNT space (close to the ends) are filled by DOX molecules. Graphical illustration of such architecture is presented in Fig. 6 for both cases of the neutral and acidic pH. Fabrication of such types of systems is probably possible by applying sequential impregnation of the nanotubes by BCNU and next DOX and finally linking the prefilled nanotubes with the iM molecules. For now, however, let us focus on possible properties of such kind of systems which can be predicted from molecular modeling.

Visual analysis of systems in Fig. 6, where DOX additionally corks the entrances to the nanotube, leads to the conclusion that their behaviors are actually very similar to those in Fig. 4. Thus, at neutral pH the system is stable and no qualitative changes of its structure can be observed within a long 100 ns simulation time. We can also predict that even in the macroscopic timescale the system at the neutral pH will preserve the same structure, which is stable binding of DOX and BCNU within iM chains and in the CNT. This conclusion comes from analysis of potentials of mean force for DOX detachments from iM chains in both cases of pH and their associated spatial forms. Thus, as found in [1] DOX molecule needs to overcome of ca. 150 kJ mol^{-1} energetic barriers during detachment from the random coil of iM chain at the neutral pH. This means that the escape of DOX from the network of iM chains in Fig. 6 is thermodynamically blocked at the neutral pH. However, the determined potential of mean force for the acidic form of iM, that is the spatial form of i-motif, is not bigger than 50 kJ mol^{-1}, as found in [1]. Indeed, as seen in Fig. 6, in the acidic form of the system (BD2-A) DOX molecules were able to spontaneously detach from the iM fragments and move on the CNT surface or even escape to the bulk. The release of DOX from the internal space of CNT is more complex process and its likelihood will be discussed later on. At the moment we can assume that the free energy barrier against release of DOX from the internal space of CNT to the bulk should be comparable to the already determined in ref. [40] value i.e. ca. 22 kJ mol^{-1}. Some difference in the nanotube chirality (20,0) vs. (30,0) may lead to a slightly larger value of the barrier for the current system with the narrower nanotube. Thus, the final state of the system BD2-A should be with DOX molecules transferred to the bulk and the BCNU molecules kept inside the CNT but with full access to water, like in the case of systems B-N and B-A. Reaching this final state requires probably macroscopic times - unreachable in molecular dynamics simulations.

The density profiles for BD2- systems shown in Fig. 7 reveal that water access to the BCNU phase is strongly reduced when compared to previous cases. However, as can be seen there is no perfect isolation of the BCNU phase from water - some overlapping of density profiles is still visible. Very important changes appear however for the water flux. For both neutral and acidic pH the fluxes have been reduced strongly due to

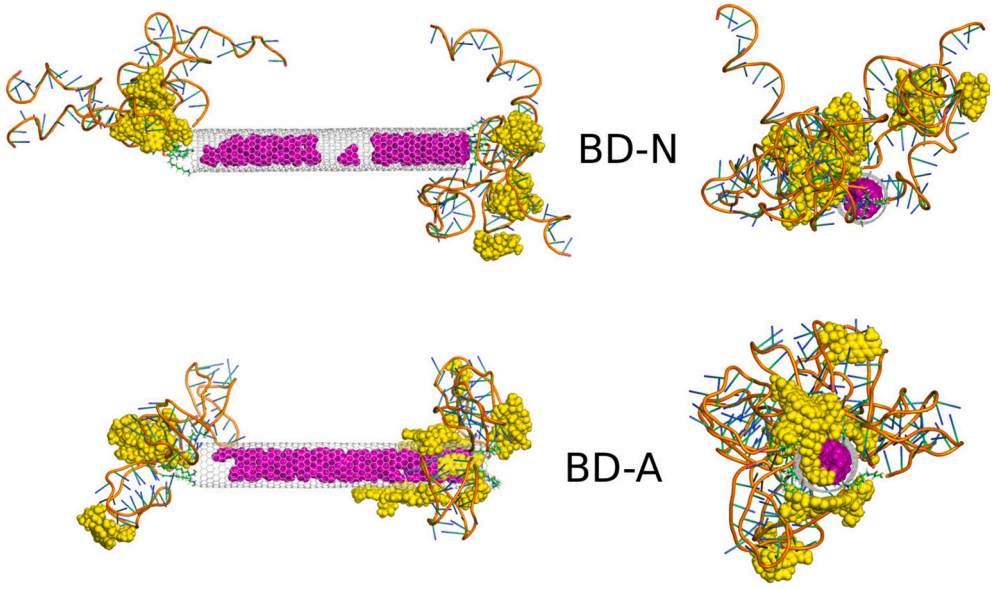

Fig. 4. Final structures of the systems BD-N and BD-A obtained after 100 ns of simulations. The colour scheme is identical as in Fig. 2 but here the systems additionally contain DOX molecules represented by the yellow spheres. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

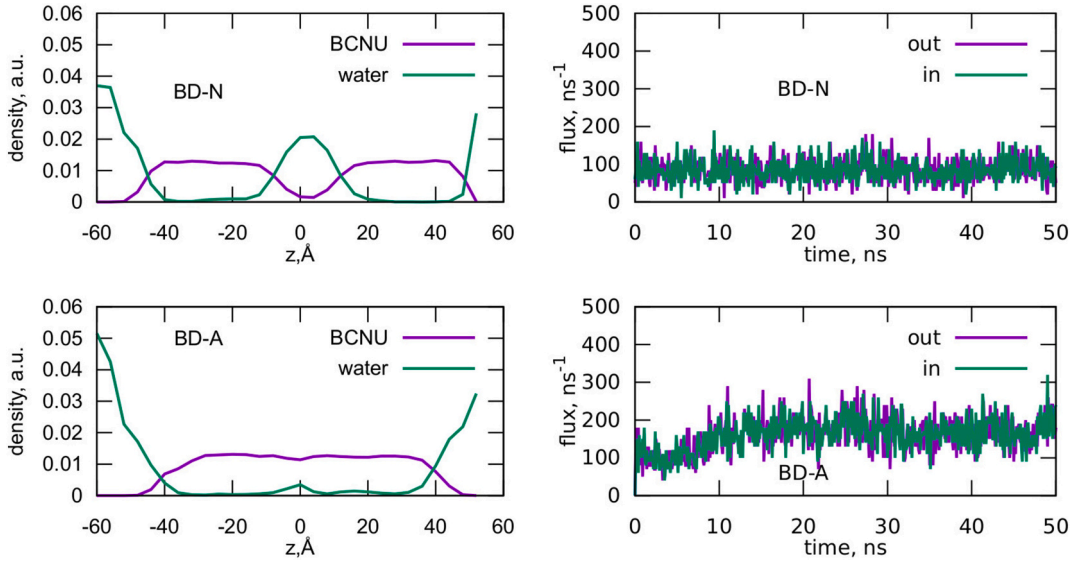

Fig. 5. Axial density profiles of BCNU molecules and water determined for the BD-N and BD-A systems (left panel) and flux of water molecules into and outside the CNT (right panel).

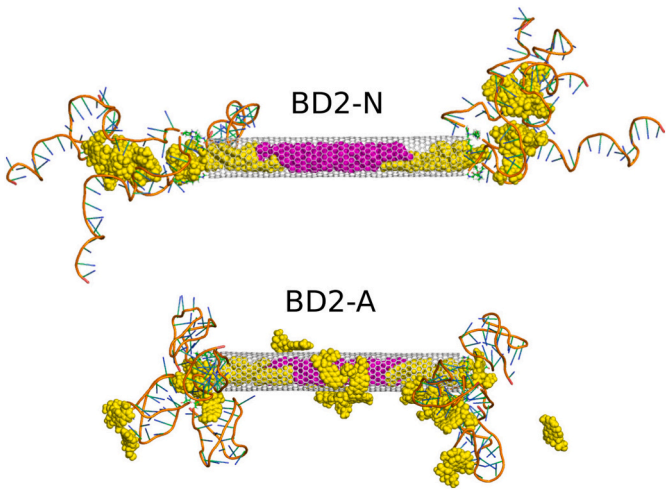

Fig. 6. Final structures of the systems BD2-N and BD2-A obtained after 100 ns of simulations. The colour scheme is identical as in Figs. 2 and 3. Here the systems additionally contain DOX molecules in the inner space of CNT, represented by the yellow spheres. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the presence of DOX clusters near the CNT ends. The reduction of water flux is more than tenfold in the case of BD2-N when compared to B-N and about fourfold for BD2-A system. There is also a large difference between BD2-N and BD2-A systems (16 vs. 54 molecules per nanosecond) which suggest ability to modulate the water flux to the nanotube by utilizing changes of pH.

The properties of BD2- systems lead to following general conclusions: (i) At the neutral pH the system is highly stable and neither DOX nor BCNU can spontaneously leave the carrier. This property is thus useful during the transportation of the carrier in bloodstream prior to reaching tumor microenvironment. However, some small access of water to BCNU phase exists anyway. (ii) After reaching the acidic pH of tumor microenvironment the folding of iM occurs and DOX should gradually be leaving the carrier. At the same time water will have better and better access to the BCNU molecules.

As already discussed BCNU quickly decomposes in water solution so we have to remember that in both BD2-N and BD2-A systems the decomposition products must be present inside the CNT because of nonzero access of water. Thus, the next step of the study is the analysis of the behavior of the BCNU decomposition products within the nanotube. As already discussed the BCNU molecule decomposes into chloroethyl isocyanate and chloroethyl diazohydroxide. The latter in turn decomposes into chloroethyl carbonium cation, which is the true alkylating agent, and nitrogen molecule and hydroxide anion.

In order to model this process within the framework of the nonreactive force field we simply converted several BCNU molecules within the BD2-A system into the mentioned species and of course adjusted the force field topology to the new system composition. Then we ran simulation with that new chemical composition of the simulation box changed only in the area of the CNT interior. To sum up we had within the CNT the following species: DOX, BCNU, water, chloroethyl isocyanate, chloroethyl carbonium cations, nitrogen molecules and hydroxide anions. That system composition mimics the BD2-A system after decomposition of a few BCNU molecules and Fig. 8A shows its state after additional 100 ns of simulation.

As can be seen in Fig. 8A the decomposition products are locked within the nanotube interior and only some permeation of nitrogen molecules seems to occur. The carbonium cations though small and hydrophilic are not able to cross the layer of DOX molecules. This is because DOX molecules are positively charged and they electrostatically hinder the motion of carbonium cations through DOX layers. The conclusion is thus that within the analyzed 100 ns timescale any transfer of BCNU decomposition products from the CNT to the bulk has not occurred. What happens at macroscopic timescale is unknown though we can safely assume that until DOX molecules are present in the nanotube the diffusion of carbonium cations will be blocked. Thus the crucial role plays doxorubicin: if it escapes from the nanotube then we can expect that the final system state will be like that shown in Fig. 8B. This state shows what happens after intentional removal of DOX from the system shown in Fig. 8A. We can clearly see that both ions, i.e. chloroethyl carbonium cation and hydroxyl anion quickly escaped to the bulk and this phenomenon occurred without any bias applied. Moreover, the rest of BCNU molecules (10) still reside in the nanotube and, what is the most striking, also the chloroethyl isocyanate molecules stay within the nanotube. This is very important observation because it offers application of carbon nanotubes as agents able to lock and isolate carbamylation agent within the CNT and, at the same time, releasing the alkylating agent to the bulk. Both coming from the decomposition of the BCNU in aqueous environment.

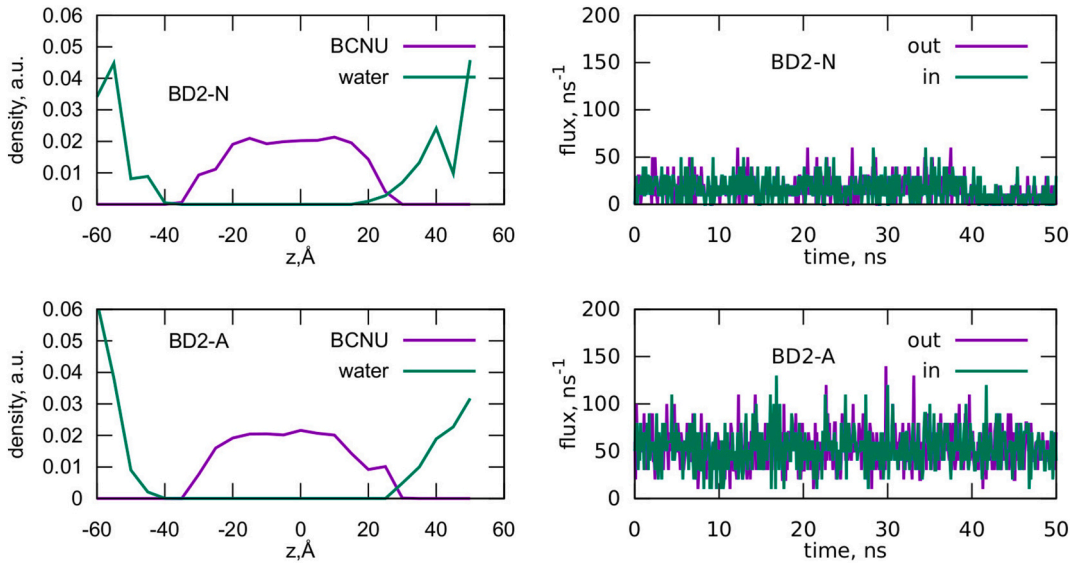

Fig. 7. Axial density profiles of BCNU molecules and water determined for the BD2-N and BD2-A systems (left panel) and flux of water molecules into and outside the CNT (right panel).

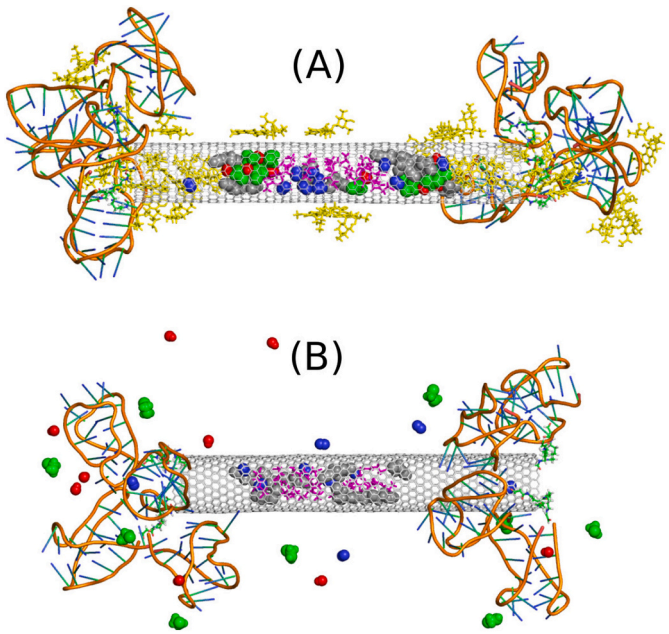

Fig. 8. (A) BD2-A system after decomposition of 8 BCNU molecules according to the scheme shown in Fig. 1. The colour code is the same as in previous figures but here there are 4 new species: chloroethyl carbonium cation (green spheres), chloroethyl isocyanate (grey spheres), nitrogen molecules (blue spheres) and hydroxyl ions (red spheres). (B) BD2-A system after removal of DOX molecules. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The release of DOX from the interior of the CNT at acidic pH is very important for the efficacy of working of the considered systems as pH controlled carriers of both DOX and BCNU. However, we have not observed such a phenomenon within the overall simulation time which reached several hundreds of nanoseconds. These are very large and complex systems and the escape of DOX molecule from the interior of CNT must follow multi stage and complex path and the process may effectively need macroscopic timescale. Of course we attempted to determine the free energy of DOX binding within the CNT in order to estimate the thermodynamic likelihood of its escape. But due to the mentioned large size and complexity of the reaction path these attempts failed due to lack of the convergence. Thus, the only parameter that could approximately describe the release phenomena of the analyzed species from the CNT interior is the work done during enforced dragging of these species out of the nanotube. These works were determined by attaching a moving spring to a given molecule and applying constant velocity pulling. Fig. 9 shows results of such calculations for chloroethyl carbonium cation (A) and chloroethyl isocyanate (B) all dragged out from the interior of the nanotube shown in Fig. 8A.

The constant velocity dragging can formally be used for determination of the free energy according to Jarzynski inequality [41]. However, it requires a number of trajectories determined from various initial configurations (all taken from canonical distribution) and averaging of work exponents which makes the overall procedure numerically very demanding even for small systems. Because the obtained work profiles shown in Fig. 9 represent only single runs from only one molecular configuration they cannot be identified with free energies. Nevertheless, analysis of these profiles and events occurring during the dragging is instructive and valuable.

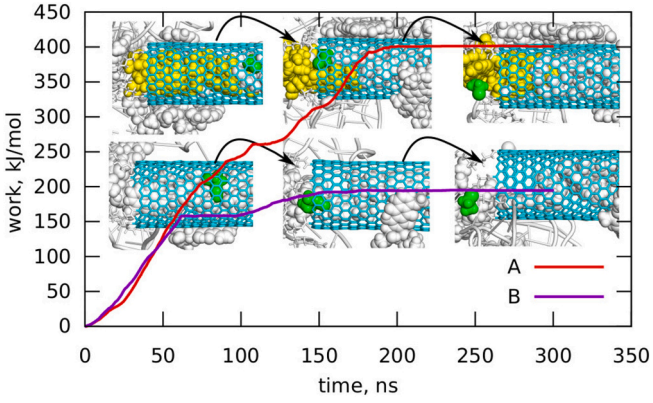

Fig. 9. Works done during enforced dragging of chloroethyl carbonium cation (A) and chloroethyl isocyanate (B) molecule from the nanotube shown in Fig. 8A. In the insets the dragged molecules are represented by green spheres: the upper panel for curve (A) and the lower panel for curve (B). The applied pulling velocity was $1.5{\cdot}10^{-4}$ Å ps^{-1} and the applied spring force constant was 50 kJ $Å^{-2}$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

First of all we can notice that dragging of carbonium cation requires large work to be done, i.e. reaching 400 kJ mol^{-1}. This is rather surprising because the picture shown in Fig. 8B suggests that carbonium cation leaves the nanotube spontaneously and quickly. The mechanism of the release of this cation can be understood from simulation snapshots shown in Fig. 9. It can be noted that dragging of that cation, which is relatively small molecule, leads to simultaneous pushing out the 4-molecule cluster of DOX (intentionally denoted as yellow spheres). In the final step the carbonium cation leaves the CNT together with at least two DOX molecules. Thus, the large work recorded during this process is actually associated with two events i.e. release of DOX and carbonium cation. This is interesting observation and its justification can be based on the assumption that both species are positively charged and the force imposed on the small cation is immediately applied also to the DOX cluster due to electrostatic repulsion between them. Thus, the carbonium cations are indeed effectively blocked inside the CNT by the presence of DOX molecules localized at its ends.

A totally different picture is observed for isocyanate molecule. This small molecule is charge neutral and the force applied during dragging leads to sliding between DOX molecules in the direction of the CNT end. The recorded work is smaller than in previous case but it still reaches ca. 190 kJ mol^{-1}. It compares well with the observation in Fig. 8B where the isocyanate species stay within the nanotube together with non-decomposed BCNU molecules and do not leave the CNT within the applied simulation time. This means that the energetic barrier against escape is in this case generated either by the presence of DOX molecules or there is also a contribution from the hydrophobic interactions with the nanotube itself. In the previous case the energetic barrier was generated mainly by the DOX molecules. It suggests that changes (increase) of nanotube diameter should not significantly affect the barrier for carbonium cation because there is no contribution from the nanotube walls. However, the isocyanate molecule should experience contribution from the nanotube even in the case of wider nanotubes.

Fig. 10, in turn, shows work profiles determined for DOX molecules. Two approaches were studied; the first one assumes dragging of the whole cluster (group) of DOX molecules localized on one side of the CNT and the second one assumes the dragging of a single DOX molecule. The processes of single molecule dragging were repeated several times using various DOX molecules localized initially in various places within the nanotube. The obtained in that way work profiles were very different in terms of maximum work accumulated or just the shape of work vs. distance profiles. The most important are of course values of work at plateau when the molecule finally exited the nanotube and those values were from 80 to 180 kJ mol^{-1}. An example of one curve together with the snapshots showing successive stages of the dragging process is shown in Fig. 10. That curve corresponds to one of the highest work values which is ca. 180 kJ mol^{-1}. However in all cases the process required breaking of attractive interaction with other molecules in the nanotube and pulling apart the bundle of DOX and iM chains molecules beyond the nanotube. The dragging of the whole group of DOX molecules proceeds in even more complex way: the pulling apart of the molecular knot localized at the nanotube entrance is the necessary step but in further stages the cluster of DOX molecules splits into two. One of them goes to the bulk but the other still stays within the nanotube. The work associated with the whole process reaches 440 kJ mol^{-1} but it can be divided into two components. The stage associated with the plateau at about 150 ns corresponds to the splitting the cluster into two parts. The rest of the curve beyond 150 ns is actually difficult to interpret since it corresponds to dragging of center mass of two independent clusters.

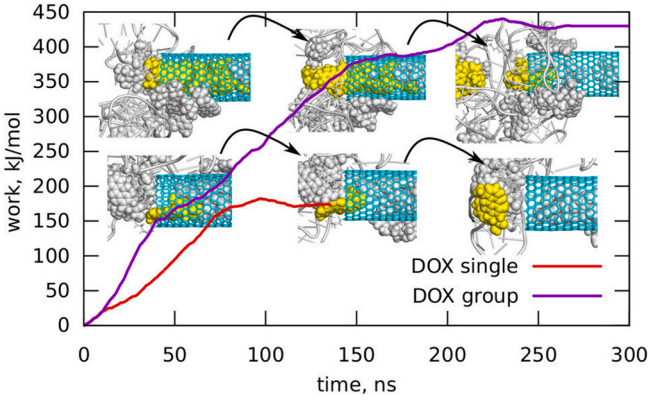

Fig. 10. Works done during enforced dragging of a single DOX molecule (red) and a group of four molecules together (violet) from the nanotube from the configuration shown in Fig. 8A. In the insets the dragged molecules are represented by yellow spheres: the upper panel is for group of DOX molecules and the lower panel is for the single DOX molecule. The applied pulling velocity was $1.5{\cdot}10^{-4}$ Å ps^{-1} and the applied spring force constant was 50 kJ Å^{-2}. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The question thus arises whether the release of DOX at acidic pH is possible due to relatively large work values obtained in nonequilibrium processes. Precise answering of this question is very difficult nevertheless we can anticipate that spontaneous escape of DOX from the CNT interior may occur after sufficiently long time and after probing many configurations of the phase space. The obtained work profiles, differing strongly in maximum work at plateau, suggest that continuation of calculations starting from other initial configurations might produce smaller work values and, according to Jarzynski inequality, the smallest value is the most representative in exponential averaging. Additionally, that conclusion can be supported by the determined free energy of DOX release from the interior of (30,0) nanotube and without any kinetic obstacles, as obtained in [40]. The presence of obstacles, like other DOX molecules entangled in iM chains at the CNT tips, kinetically hinder the motion but these obstacles must disappear because DOX spontaneously detach from i-motifs, as seen in Figs. 4, 6, and 8, and can go to the bulk. On the other hand, the binding of DOX within the nanotube can be controlled by adjusting the diameter of the nanotube. Wider nanotubes may store more water and thus DOX molecules can easier diffuse out of such nanotubes. Therefore, the presented model can be fine-tuned by adjusting the CNT diameter, length, density of iM fragments attached to the tips etc. So, finally such type of the molecular system can reveal the properties of pH controlled BCNU carrier or just allows for the control of the release of the BCNU decomposition products.

4. Summary

The performed research led us to recognition of several new physical insights related to properties of DOX and BCNU molecules interacting with carbon nanotubes. The first observation was a very stable binding of BCNU molecules inside nanotubes and totally free access of water molecules to the BCNU phase inside the CNT. The access of water to BCNU and possible decomposition of the BCNU molecules was found to be controllable to some extent by the presence of DOX molecules localized within the iM areas of the system. The water flux was found to be dependent on pH or more precisely on the spatial shape of iM fragments which together with DOX formed stiff or soft areas at the CNT interiors.

The most interesting features of the considered system were observed when some small amount of DOX molecules was incorporated to the CNT. Then, the system was found as more sensitive to pH in terms of water flux into the BCNU phase localized in the CNT interior. Further analysis focused on the behavior of BCNU decomposition products led to important conclusion that though the decomposition products are small molecules or ions they are still blocked against release by the DOX molecules. Both chloroethyl carbonium cation and chloroethyl isocyanate were blocked by the DOX molecules but after intentional removal of DOX from the system only the chloroethyl carbonium cation spontaneously and quickly left the nanotube. This is the true alkylating agent and

because the second component i.e. chloroethyl isocyanate was still kept within the nanotube we concluded that carmustine decomposition products can be naturally divided inside the nanotube and only the alkylating component can be released while the carbamylating agent is still isolated from the environment. This is very interesting and useful property of BCNU decomposition occurring in the inner space of carbon nanotubes.

Finally, we found that DOX molecules are kept inside the nanotube the least strongly when compared to the other species, as found in analysis of works associated with enforced dragging of those species out of the nanotube. Combining various sources of information about binding of DOX inside the nanotube we concluded that it is possible to tune the system parameters in such a way that DOX will be spontaneously released in the acidic pH. Then the release of chloroethyl carbonium cation will occur but chloroethyl isocyanate molecule will still be blocked inside the CNT. The predicted phenomena deserve further extensive studies including the experimental trials as the described properties are potentially useful in designing of novel smart drug carriers.

CRediT authorship contribution statement

Pawel Wolski: Conceptualization, Methodology, Investigation, Visualization, Writing – review & editing. **Krzysztof Nieszporek:** Validation, Formal analysis, Investigation, Data curation. **Tomasz Panczyk:** Conceptualization, Methodology, Investigation, Visualization, Software, Writing – original draft, 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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Appendix A. Supplementary data

The provided files are the force field topology files for all constituents of the analyzed systems. The files ‘acidic.top’ and ‘neutral.top’ are the force field topologies for the whole constructs composed of carbon nanotube, spacer molecules and C-rich DNA fragments in folded into i-motif/protonated state and unfolded/uprotonated state, respectively. The files ‘DOX.itp’, ‘BCNU.itp’, ‘OCNCH2CH2CL.itp’ and ‘CH2CH2Cl.itp’ are the force field topologies for the other small molecules used in the computations. The associated gro files are atomic coordinate files for the considered molecules. Supplementary data to this article can be found online at doi: https://doi.org/10.1016/j.bioadv.2022.212835.

References

[1] P. Wolski, K. Nieszporek, T. Panczyk, Carbon nanotubes and short cytosine-rich telomeric DNA oligomeres as platforms for controlled release of doxorubicin—a molecular dynamics study, IJMS 21 (2020) 3619, https://doi.org/10.3390/ijms21103619.
[2] P. Wolski, K. Nieszporek, T. Panczyk, Cytosine-rich DNA fragments covalently bound to carbon nanotube as factors triggering doxorubicin release at acidic pH.A molecular dynamics study, IJMS 22 (2021) 8466, https://doi.org/10.3390/ijms22168466.
[3] Z. Li, A.L.B. de Barros, D.C.F. Soares, S.N. Moss, L. Alisaraie, Functionalized single-walled carbon nanotubes: cellular uptake, biodistribution and applications in drug delivery, Int. J. Pharm. 524 (2017) 41–54, https://doi.org/10.1016/j.ijpharm.2017.03.017.
[4] N.K. Mehra, S. Palakurthi, Interactions between carbon nanotubes and bioactives: a drug delivery perspective, Drug Discov. Today 21 (2016) 585–597, https://doi.org/10.1016/j.drudis.2015.11.011.
[5] M.I. Sajid, U. Jamshaid, T. Jamshaid, N. Zafar, H. Fessi, A. Elaissari, Carbon nanotubes from synthesis to in vivo biomedical applications, Int. J. Pharm. 501 (2016) 278–299, https://doi.org/10.1016/j.ijpharm.2016.01.064.
[6] A. Battigelli, C. Ménard-Moyon, T. Da Ros, M. Prato, A. Bianco, Endowing carbon nanotubes with biological and biomedical properties by chemical modifications, Adv. Drug Deliv. Rev. 65 (2013) 1899–1920, https://doi.org/10.1016/j.addr.2013.07.006.
[7] M. Karimi, N. Solati, A. Ghasemi, M.A. Estiar, M. Hashemkhani, P. Kiani, E. Mohamed, A. Saeidi, M. Taheri, P. Avci, A.R. Aref, M. Amiri, F. Baniasadi, M. R. Hamblin, Carbon nanotubes part II: a remarkable carrier for drug and gene delivery, Expert Opin.Drug Deliv. 12 (2015) 1089–1105, https://doi.org/10.1517/17425247.2015.1004309.
[8] A. Jagusiak, J. Goclon, T. Panczyk, Adsorption of Evans blue and Congo red on carbon nanotubes and its influence on the fracture parameters of defective and functionalized carbon nanotubes studied using computational methods, Appl. Surf. Sci. 539 (2021), 148236, https://doi.org/10.1016/j.apsusc.2020.148236.
[9] J. Goclon, T. Panczyk, K. Winkler, Investigation of the interfacial properties of polyurethane/carbon nanotube hybrid composites: a molecular dynamics study, Appl. Surf. Sci. 433 (2018) 213–221, https://doi.org/10.1016/j.apsusc.2017.09.192.
[10] B.S. Wong, S.L. Yoong, A. Jagusiak, T. Panczyk, H.K. Ho, W.H. Ang, G. Pastorin, Carbon nanotubes for delivery of small molecule drugs, Adv. Drug Deliv. Rev. 65 (2013) 1964–2015, https://doi.org/10.1016/j.addr.2013.08.005.
[11] S. Luanpitpong, L. Wang, V. Castranova, C.Z. Dinu, S. Issaragrisil, Y.C. Chen, Y. Rojanasakul, Induction of cancer-associated fibroblast-like cells by carbon nanotubes dictates its tumorigenicity, Sci. Rep. 6 (2016), https://doi.org/10.1038/srep39558.
[12] C.A. Leyva-González, D. Salas-Treviño, F.F. Contreras-Torres, M. de J. Loera-Arias, C.A. Gómez-Tristán, E.I. Piña-Mendoza, G. de J. García-Rivas, G.A. Guillén-Meléndez, R. Montes-de-Oca-Luna, O. Saucedo-Cárdenas, A. Soto-Domínguez, Hyaluronate functionalized multi-wall carbon nanotubes loaded with carboplatin enhance cytotoxicity on human cancer cell lines, Materials 14 (2021) 3622, https://doi.org/10.3390/ma14133622.
[13] G. Pastorin, Crucial functionalizations of carbon nanotubes for improved drug delivery: a valuable option? Pharm. Res. 26 (2009) 746–769, https://doi.org/10.1007/s11095-008-9811-0.
[14] L. Niu, L. Meng, Q. Lu, Folate-conjugated PEG on single walled carbon nanotubes for targeting delivery of doxorubicin to cancer cells: folate-conjugated PEG on single walled carbon nanotubes …, Macromol. Biosci. 13 (2013) 735–744, https://doi.org/10.1002/mabi.201200475.
[15] A. Solhjoo, Z. Sobhani, A. Sufali, Z. Rezaei, S. Khabnadideh, A. Sakhteman, Exploring pH dependent delivery of 5-fluorouracil from functionalized multi-walled carbon nanotubes, Colloids Surf. B: Biointerfaces 205 (2021), 111823, https://doi.org/10.1016/j.colsurfb.2021.111823.
[16] C.L. Lay, J. Liu, Y. Liu, Functionalized carbon nanotubes for anticancer drug delivery, Expert Rev.Med.Devices 8 (2011) 561–566, https://doi.org/10.1586/erd.11.34.
[17] A.T. Phan, J.-L. Mergny, Human telomeric DNA: G-quadruplex, i-motif and Watson-Crick double helix, Nucleic Acids Res. 30 (2002) 4618–4625.
[18] A. Dembska, The analytical and biomedical potential of cytosine-rich oligonucleotides: a review, Anal. Chim. Acta 930 (2016) 1–12, https://doi.org/10.1016/j.aca.2016.05.007.
[19] C. Chen, F. Pu, Z. Huang, Z. Liu, J. Ren, X. Qu, Stimuli-responsive controlled-release system using quadruplex DNA-capped silica nanocontainers, Nucleic Acids Res. 39 (2011) 1638–1644, https://doi.org/10.1093/nar/gkq893.
[20] L. Chen, J. Di, C. Cao, Y. Zhao, Y. Ma, J. Luo, Y. Wen, W. Song, Y. Song, L. Jiang, A pH-driven DNA nanoswitch for responsive controlled release, Chem. Commun. 47 (2011) 2850, https://doi.org/10.1039/c0cc04765a.
[21] L. Song, V.H.B. Ho, C. Chen, Z. Yang, D. Liu, R. Chen, D. Zhou, Efficient, pH-triggered drug delivery using a pH-responsive DNA-conjugated gold nanoparticle, Adv.Healthc.Mater. 2 (2013) 275–280, https://doi.org/10.1002/adhm.201200112.
[22] C. Xu, C. Zhao, J. Ren, X. Qu, pH-controlled reversible drug binding and release using a cytosine-rich hairpin DNA, Chem. Commun. 47 (2011) 8043, https://doi.org/10.1039/c1cc12594j.
[23] J.Y. Kim, J. Song, H. Jung, H. Mok, I-motif-coated exosomes as a pH-sensitive carrier for anticancer drugs, Appl.Biol. Chem. 61 (2018) 599–606, https://doi.org/10.1007/s13765-018-0394-0.
[24] H. Park, J. Kim, S. Jung, W.J. Kim, DNA-Au nanomachine equipped with i-motif and G-quadruplex for triple combinatorial anti-tumor therapy, Adv. Funct. Mater. 28 (2018) 1705416, https://doi.org/10.1002/adfm.201705416.
[25] W. Wu, J.L. Klockow, M. Zhang, F. Lafortune, E. Chang, L. Jin, Y. Wu, H. E. Daldrup-Link, Glioblastoma multiforme (GBM): an overview of current therapies and mechanisms of resistance, Pharmacol. Res. 171 (2021), 105780, https://doi.org/10.1016/j.phrs.2021.105780.
[26] W.P. Tong, M.C. Kirk, D.B. Ludlum, Formation of the cross-link 1-[N3-deoxycytidyl),2-[N1-deoxyguanosinyl]ethane in DNA treated with N, N'-bis(2-chloroethyl)-N-nitrosourea, Cancer Res. 42 (1982) 3102–3105.
[27] A. Lemoine, C. Lucas, R.M.J. Ings, Metabolism of the chloroethylnitrosoureas, Xenobiotica 21 (1991) 775–791, https://doi.org/10.3109/00498259109039517.
[28] M.D. Bacolod, S.P. Johnson, A.E. Pegg, M.E. Dolan, R.C. Moschel, N.S. Bullock, Q. Fang, O.M. Colvin, P. Modrich, D.D. Bigner, H.S. Friedman, Brain tumor cell lines resistant to O6-benzylguanine/1,3-bis(2-chloroethyl)-1-nitrosourea chemotherapy have O6-alkylguanine-DNA alkyltransferase mutations, Mol. Cancer Ther. 3 (2004) 1127–1135.

[29] P. Wolski, J. Narkiewicz-Michalek, M. Panczyk, G. Pastorin, T. Panczyk, Molecular dynamics modeling of the encapsulation and de-encapsulation of the carmustine anticancer drug in the inner volume of a carbon nanotube, J. Phys. Chem. C 121 (2017) 18922–18934, https://doi.org/10.1021/acs.jpcc.7b05229.
[30] S.S. Begum, D. Das, N.K. Gour, R.C. Deka, Computational modelling of nanotube delivery of anti-cancer drug into glutathione reductase enzyme, Sci. Rep. 11 (2021) 4950, https://doi.org/10.1038/s41598-021-84006-1.
[31] G. Sánchez-Pomales, L. Santiago-Rodríguez, C.R. Cabrera, DNA-functionalized carbon nanotubes for biosensing applications, J. Nanosci. Nanotech. 9 (2009) 2175–2188, https://doi.org/10.1166/jnn.2009.SE47.
[32] J. Wang, R.M. Wolf, J.W. Caldwell, P.A. Kollman, D.A. Case, Development and testing of a general amber force field, J. Comput. Chem. 25 (2004) 1157–1174, https://doi.org/10.1002/jcc.20035.
[33] J. Wang, W. Wang, P.A. Kollman, D.A. Case, Automatic atom type and bond type perception in molecular mechanical calculations, J. Mol. Graph. Model. 25 (2006) 247–260, https://doi.org/10.1016/j.jmgm.2005.12.005.
[34] A.W. Sousa da Silva, W.F. Vranken, ACPYPE - AnteChamber PYthon parser interfacE, BMC Res.Notes 5 (2012) 367, https://doi.org/10.1186/1756-0500-5-367.
[35] S.J. Weiner, P.A. Kollman, D.T. Nguyen, D.A. Case, An all atom force field for simulations of proteins and nucleic acids, J. Comput. Chem. 7 (1986) 230–252, https://doi.org/10.1002/jcc.540070216.
[36] I. Ivani, P.D. Dans, A. Noy, A. Pérez, I. Faustino, A. Hospital, J. Walther, P. Andrio, R. Goñi, A. Balaceanu, G. Portella, F. Battistini, J.L. Gelpí, C. González, M. Vendruscolo, C.A. Laughton, S.A. Harris, D.A. Case, M. Orozco, Parmbsc1: a refined force field for DNA simulations, Nat. Methods (2015), https://doi.org/10.1038/nmeth.3658.
[37] F.-Y. Dupradeau, A. Pigache, T. Zaffran, C. Savineau, R. Lelong, N. Grivel, D. Lelong, W. Rosanski, P. Cieplak, The R.E.D. tools: advances in RESP and ESP charge derivation and force field library building, Phys. Chem. Chem. Phys. 12 (2010) 7821, https://doi.org/10.1039/c0cp00111b.
[38] E. Vanquelef, S. Simon, G. Marquant, E. Garcia, G. Klimerak, J.C. Delepine, P. Cieplak, F.-Y. Dupradeau, R.E.D. Server, A web service for deriving RESP and ESP charges and building force field libraries for new molecules and molecular fragments, Nucleic Acids Res. 39 (2011) W511–W517, https://doi.org/10.1093/nar/gkr288.
[39] H.J.C. Berendsen, D. van der Spoel, R. van Drunen, GROMACS: a message-passing parallel molecular dynamics implementation, Comput. Phys. Commun. 91 (1995) 43–56, https://doi.org/10.1016/0010-4655(95)00042-E.
[40] T. Panczyk, P. Wolski, L. Lajtar, Coadsorption of doxorubicin and selected dyes on carbon nanotubes.Theoretical investigation of potential application as a pH-controlled drug delivery system, Langmuir 32 (2016) 4719–4728, https://doi.org/10.1021/acs.langmuir.6b00296.
[41] C. Jarzynski, Nonequilibrium equality for free energy differences, Phys. Rev. Lett. 78 (1997) 2690–2693, https://doi.org/10.1103/PhysRevLett.78.2690.