Unraveling the interaction between doxorubicin and DNA origami nanostructures for customizable chemotherapeutic drug release

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Doxorubicin (DOX) is a commonly employed drug in cancer chemotherapy, and its high DNA-binding affinity can be harnessed in preparing DOX-loaded DNA nanostructures for targeted delivery and therapeutics. Although DOX has been widely studied, the existing literature of promising DOX-loaded DNA nanocarriers remains limited and incoherent. Here, based on an in-depth spectroscopic analysis, we characterize and optimize the DOX loading into different 2D and 3D scaffolded DNA origami nanostructures. In our experimental conditions, all of our DNA origami designs show similar DOX binding capacities, which are, however, remarkably lower than previously reported. To simulate the possible physiological degradation pathways, we examine the stability and DOX release properties of the complexes upon DNase I digestion, revealing customizable drug release profiles related to the DNA origami superstructure and the loaded DOX content. In addition, we identify major DOX aggregation mechanisms and spectral changes linked to pH, magnesium, and DOX concentration that have been largely ignored in experimenting with DNA nanostructures. Therefore, we believe this work can act as a guide to tailoring the release profiles and developing better drug delivery systems based on DNA carriers.

DNA nanotechnology | DNA origami | drug delivery | intercalation | groove binding | drug release | stability | nucleases | enzymatic digestion

Introduction

The possibility to employ DNA molecules in engineering artificial nanostructures (1, 2) has drawn increasing attention during the past two decades (3–5). The intense development of DNA nanotechnology has yielded new methods to build user-defined nano-objects (6), such as DNA origami (7–14), for a variety of scientific and technological uses (15–18). In particular, these custom DNA nanoshapes show considerable promise in biomedicine and drug delivery (19–22). Rationally designed DNA nanovehicles can encapsulate and display selected cargoes (23–26), act as therapeutics themselves (27), serve as platforms for various targeting ligands and tailored nucleic acid sequences (28, 29), or directly host diverse DNA-binding drugs (30, 31). In the latter case, the most frequently used drug is anthracycline doxorubicin (DOX), a fluorescent DNA-intercalator, which is applied in the treatments of several cancer types and primarily in solid tumor growth suppression (32). Its mechanisms of anticancer and cardiotoxic actions are not fully understood, as it inhibits human type IIA DNA topoisomerase function either by poisoning the catalytic activity or preventing DNA-binding. However, it also affects multiple cellular processes through DNA intercalation and its ability to generate reactive oxygen species (ROS) (33). There are a number of reports, in which the properties and therapeutic potency of various DOX-loaded DNA nanostructures have been demonstrated using *in vitro* and *in vivo* models (34–45).

Typically, the presumed intercalation and release of DOX are characterized using straightforward spectroscopic indicators such as spectral changes of visible light absorption or DOX fluorescence quenching upon DNA binding interaction. However, besides intercalation, DOX may also be complexed with DNA through (pre-intercalation) minor-groove binding and stacking into aggregates depending on the DNA sequence, prevalent DOX concentration and experimental conditions such as the ionic strength of the solution (46–48). Spectroscopic features of DOX – *e.g.* the extent of fluorescence quenching – are likewise dependent on the mode of interaction. In addition, a recent study suggests that in some cases the accessibility of the intercalation binding sites in DNA nanostructures may be restricted (49). DOX molecules have two distinct protonation states within a physiologically relevant pH range (pH ∼4–9) and they are prone to self-aggregation at high concentrations (50). Therefore, spectroscopic properties of DOX are also subject to change in different media compositions. Overlooking the multiple factors, such as the binding site availability, DOX solubility, concentration, chosen loading and purification methods, ionic strength and pH of the solution may result in misleading interpretations of DOX loading capacity, release efficiency and thus also the therapeutic effect (22).

In this work, we systematically study the binding of DOX to five structurally distinct two- (2D) and three-dimensional (3D) scaffolded DNA origami shapes (one exemplary DNA origami design shown in Figure 1). By means of absorption, excitation and fluorescence spectroscopy techniques, we optimize the loading process and uncover the contributions of the ionic strength, pH, and DOX concentration to the mea-

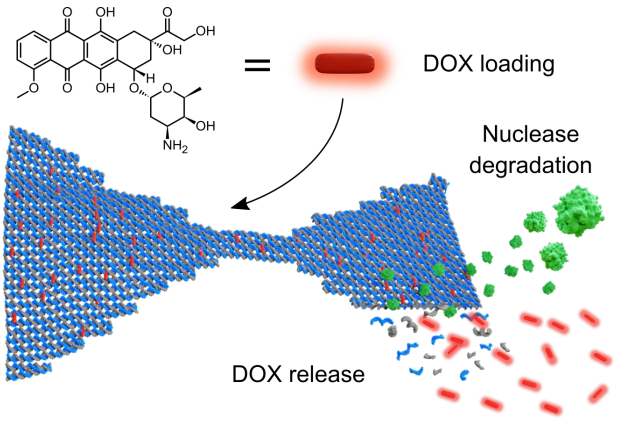

Figure 1. Schematics of the doxorubicin (DOX) loading into a DNA nanostructure and subsequent release upon enzymatic degradation. DOX molecules (red) are loaded into/onto a bowtie DNA origami (formed from blue and grey strands), and the process is optimized by monitoring the spectroscopic features of DOX, such as fluorescence quenching. The DOX is sustainedly released as DNA origami is subjected to nuclease (DNase I, green) degradation and digested into single-stranded DNA fragments. Real-time spectroscopic observation reveals that the degradation rate of DNA nanostructures and the release profiles of DOX depend on DNA origami superstructure and the applied DOX content.

sured spectra. The obtained results reveal that the DOX binding capacity of DNA origami has often been substantially overestimated, in some previously reported cases by more than two orders of magnitude.

Finally, we mimic one plausible and physiologically relevant DOX release pathway by subjecting the DOX-loaded DNA origami to deoxyribonuclease I (DNase I) digestion (see Figure 1) (51–53). Real-time monitoring of the spectroscopic changes during the digestion show that both the DNA degradation rates and the DOX release profiles depend on the DNA origami superstructure and the amount of DOX loaded. We believe that by unraveling all these fundamental and some previously undiscovered features, the engineering of new tailored DNA objects with imminent biomedical potential becomes feasible. Through identification of the loading, release and spectroscopic properties of DOX, as well as the superstructure-dependent stability factors of DNA origami in physiological conditions (54–57), it may become possible to rationally design delivery capability, control the dose and thus achieve the optimal therapeutic efficacy.

Results

Effects of buffer conditions on the spectroscopic features of DOX

To ensure that the obtained spectroscopic changes in later experiments are associated reliably with the DOX-DNA binding events and not caused by the environment, we first identified the effects of the buffer conditions on the spectroscopic properties of DOX. We performed a series of measurements on DOX in the absence of DNA in Tris-based buffers typically applied in DNA origami experiments. In particular, we screened the effect of two buffer parameters; pH and $MgCl_2$ concentration.

Buffer pH. For identifying the effects of buffer pH on the spectroscopic features of DOX, 40 mM Tris-HCl buffers were prepared at pH 6.0–9.0 and the absorption and fluorescence spectra of DOX were collected at each pH. The shape of the DOX absorption spectrum as well as its molar extinction coefficient (ϵ) depends heavily on buffer pH (Figure 2a). Between pH 6.0–8.0, the shape of the spectrum is maintained, but throughout the whole absorption spectrum, ϵ increases with decreasing pH. For instance, ϵ_{494} is *ca.* 65% higher at pH 6.0 than at pH 8.0. A higher emission intensity is also observed at lower pH values, as shown in Figure 2a inset with a 494 nm excitation.

Above pH 8.0, the shape of the absorption spectrum changes and a new absorption peak emerges at approximately 590 nm. However, exciting the molecules at this wavelength does not lead to DOX fluorescence, thus showing that at pH 8.0 and above, an increasing fraction of DOX molecules is non-fluorescent. DOX is known to have a pK_a value for the deprotonation of the amino sugar NH_3^+ group at pH 8.2 (50). The observed spectral changes and the emergence of non-fluorescent molecules take place around the same pH value, being thus likely associated with the deprotonation events. These observations are also in line with previous reports of DOX absorbance in high pH buffers (58), and the spectral changes could thus be expected to become even more pronounced at pH values above 9.0.

Near the pK_a, the sample contains a distribution of charged and neutral molecules, and in the spectroscopic means, a mixture of fluorescent and non-fluorescent DOX molecules. While the sample is thus heterogeneous, the emission spectrum remains homogeneous as the non-fluorescent molecules do not contribute to the signal (Supplementary Figure 1). As the sample heterogeneity would nevertheless complicate the interpretation of experimental results, it is beneficial to conduct experiments at pH well below the pK_a. Based on both the existing literature and the obtained spectra, an optimal pH range for further experiments was determined as 6.0–7.8, where altering the pH does not change the shape of the absorption spectrum.

Buffer $MgCl_2$ concentration at pH 7.4. DOX is known to form complexes with metal ions, such as Fe^{3+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}, Mg^{2+}, and Zn^{2+} (58–60). Metal ion complexation thus presents another source of DOX heterogeneity in buffers supplemented with divalent cations. When the $MgCl_2$ concentration in the buffer increases, both the absorption and fluorescence properties of DOX change indicating complexation of DOX with Mg^{2+} ions (Figure 2b). In the presence of 100 mM $MgCl_2$, three distinct peaks at 500 nm, 534 nm, and 576 nm are observed in the absorption spectrum. The 576 nm peak emerges only in the presence of $MgCl_2$, and excitation at this absorption peak leads to a fluorescence spectral shape that is rather distinct from that of DOX in the absence of $MgCl_2$ (Supplementary Figure 2). While the emission spectrum of DOX at 0 mM $MgCl_2$ is homogeneous over the full absorption spectrum, the addition of $MgCl_2$ induces heterogeneity in the emission measurement reflected as the shape of the emission spectrum changing with the excitation wave-

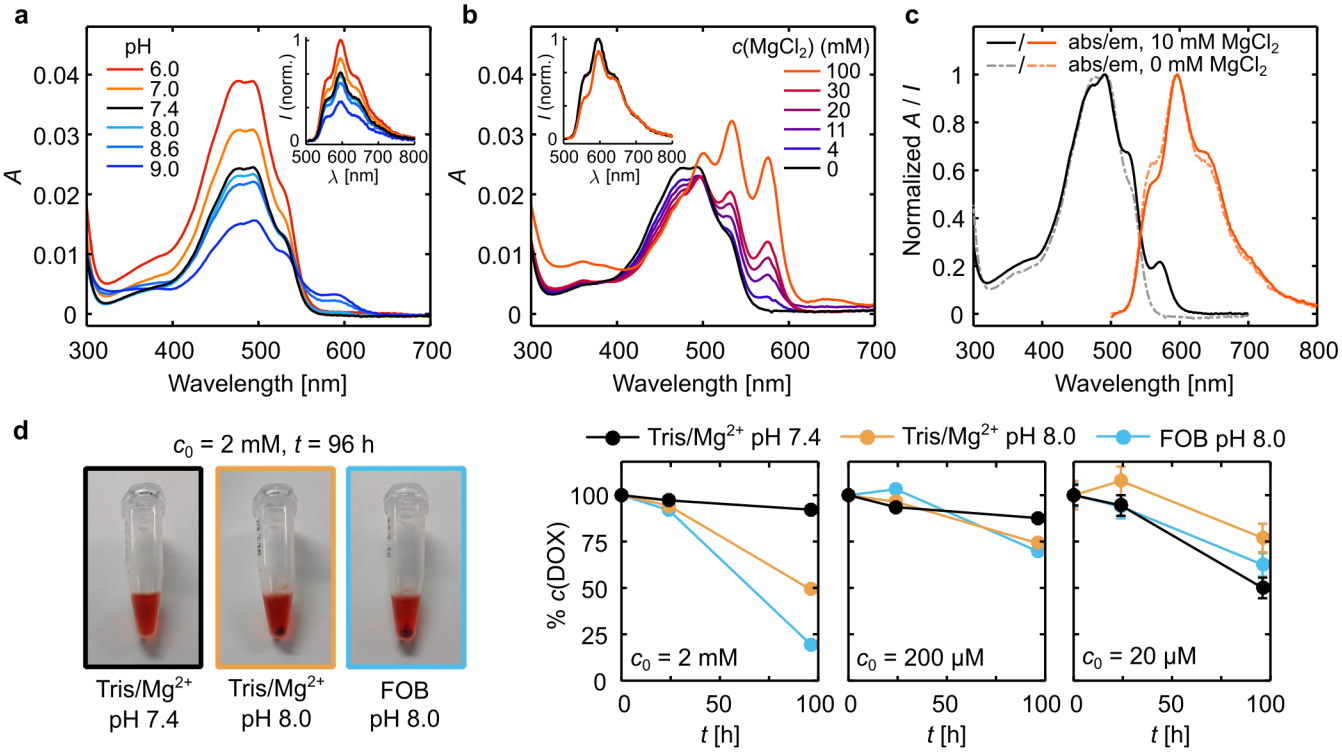

Figure 2. Effects of buffer conditions on the properties of the DOX in the absence of DNA. **a** Absorption and emission (inset) spectra of 3 μM DOX in 40 mM Tris, 0 mM $MgCl_2$ at pH 6.0–9.0. The emission spectra were obtained at an excitation wavelength of 494 nm. **b** Absorption and fluorescence emission (494 nm excitation) of 3 μM DOX in 40 mM Tris, pH 7.4 buffer at different $MgCl_2$ concentrations. The inset figure shows a comparison of the emission spectra of the 0 mM and 100 mM samples, with the maximum emission intensity of the 0 mM $MgCl_2$ sample normalized to 1. The 0 mM $MgCl_2$ spectrum (black) corresponds to the pH 7.4 spectrum in Figure a. **c** Absorption and emission spectra of 3 μM DOX in the chosen experimental conditions – 40 mM Tris, 10 mM $MgCl_2$, pH 7.4. The effect of 10 mM $MgCl_2$ concentration is shown by comparing the spectra measured at 10 mM $MgCl_2$ concentration (solid lines) with spectra measured at 0 mM $MgCl_2$ (dashed lines). **d** Precipitation of DOX driven by high DOX concentration (mM scale), high pH (8.0), and increasing magnesium:DOX ratio (12.5 mM Mg^{2+} in FOB and 10 mM Mg^{2+} in both Tris/Mg^{2+} buffers, DOX concentration varies as indicated). The photographs on the left show the visible precipitation of 2 mM DOX in pH 8.0 buffers observed after 96 h incubation at RT and centrifugation at 14,000 g. The graphs on the right show the effect of the incubation time, total DOX concentration in the beginning of the incubation (c_0) and the applied buffer to the extent of self-aggregation and precipitation of DOX. The magnesium:DOX ratio in the samples increases from left to right with decreasing c_0. The concentration of DOX remaining in the supernatant (% c(DOX)) was quantified with absorbance (A_{480}) measurements.

length (Supplementary Figure 2). As a result, the shape of the emission spectrum upon 494 nm excitation depends slightly on the $MgCl_2$ concentration (Figure 2b inset).

A comparison of the absorption and fluorescence spectra of 3 μM DOX in 40 mM Tris, pH 7.4 with either 0 mM or 10 mM $MgCl_2$ is shown in Figure 2c. The spectral differences indicate that at 10 mM $MgCl_2$, the sample and its absorption and fluorescence spectra are a combination of pure DOX and a small concentration of the DOX-Mg^{2+} complex. Despite the slight DOX heterogeneity in these conditions, 40 mM Tris at pH 7.4 supplemented with 10 mM $MgCl_2$ was chosen for all the experiments to maintain structural stability and integrity of the DNA origami.

DOX self-aggregation in high-pH buffers and precipitation due to Mg^{2+} ions over time

We then tested whether the effects of pH and $MgCl_2$ observed as spectral changes can also change the behavior of DOX in an extent that could affect the outcome of DOX - DNA origami experiments. In particular, the combined effect of pH and the applied DOX concentration can be expected to be large: near and above the pK_a of the NH_3^+ group, the solubility of the uncharged form of DOX is only 0.3 mg/mL (0.55 mmol/L; M = 543.52 g/mol), while the solubility of positively charged DOX in aqueous solution has been reported as 20 mg/mL (36.8 mmol/L) (50). In addition, high DOX concentrations can lead to dimerization (K_a = 1.4×10^4 M^{-1}) (47) or oligomerization of DOX (50). Nevertheless, DOX-loaded DNA origami structures are often prepared using DOX concentrations within the range of 1–2 mM at pH 8.0 or above (34, 36, 39, 41).

To characterize the effect of different experimental conditions, we studied the extent of self-aggregation of 20 μM to 2 mM DOX solutions prepared in three different buffers: in 40 mM Tris, 10 mM $MgCl_2$ at pH 7.4; in 40 mM Tris, 10 mM $MgCl_2$ at pH 8.0; and in a typical 2D DNA origami folding buffer (FOB) containing 1× TAE [40 mM Tris, 19 mM acetic acid, 1 mM ethylenediaminetetraacetic acid (EDTA)] and 12.5 mM $MgCl_2$ at pH 8.0. After incubating the DOX solutions in the dark at room temperature, centrifugation was used to separate the insoluble fraction of DOX formed during the incubation.

At 2 mM DOX concentration, the choice of buffer shows a significant effect on the extent of DOX precipitation during incubation. After 96-hour incubation, centrifugation leads to a formation of a dark red DOX precipitate in both of the

2 mM DOX samples prepared in pH 8.0 buffers, but not in the pH 7.4 buffer (Figure 2d, photographs on the left panel). The observed DOX precipitation was further confirmed and quantified by determining the concentration of DOX in the supernatant from DOX absorbance (graphs in Figure 2d). At 2 mM DOX concentration in FOB, only 0.4 mM of DOX remains soluble after 96 hours. In Tris/Mg^{2+} at the same pH 8.0, but containing slightly less $MgCl_2$, the concentration of DOX in the supernatant is 1.0 mM. In the Tris/Mg^{2+} buffer at pH 7.4, aggregation is negligible with 1.8 mM of DOX found in the supernatant.

When the DOX concentration is decreased to 200 μM, *i.e*. below the solubility limit of the deprotonated DOX molecules, the differences between the buffers become smaller. However, when the relative amount of magnesium to DOX increases (from left to right in Figure 2d), DOX precipitation due to the apparent DOX-magnesium interaction starts to play a significant role. This effect is clearly seen at 20 μM DOX after a long-term storage. Comparison of the full absorption spectra of the samples likewise shows that the effect of Mg^{2+} on the shape of the spectra increases at lower DOX concentrations where the relative amount of magnesium is large (Supplementary Figure 3).

DOX loading

Next, we studied the interaction between DOX and five structurally distinct DNA origami nanostructures in the selected buffer conditions. The DNA origami structures include three 2D and two 3D DNA origami designs (Figure 3a): the Rothemund triangle (7), a bowtie (61), a double-L (61), a capsule (26), and a 24-helix bundle (24HB) (see Supplementary Figures 12–14 and Supplementary Table 5). The correct high-yield folding and structural integrity of all the DNA origami structures were verified with atomic force microscopy (AFM) or transmission electron microscopy (TEM) (Figure 3a).

Absorption and fluorescence properties of the DOX-DNA origami complexes. To study the DOX loading process in detail, we performed a set of titration experiments where the concentration of DOX was held constant at 3 μM while the concentration of DNA origami in the sample was increased. When the concentration of DNA in the sample increases over the titration, an increasing fraction of DOX binds to DNA, observed as changes in DOX light absorption and quenching of fluorescence. After addition of DNA, the samples were incubated for 2.5 min before the spectra were collected in order to reach an equilibrium where the amount of DOX bound to the DNA has stabilized. The 2.5 min incubation time was found to be sufficient, as no further spectral changes were observed over longer incubation times (Supplementary Figure 9), in accordance with existing literature where the equilibrium is reached within seconds (47).

The left panel of Figure 3b shows how the absorption spectrum of DOX changes upon titration with the triangle DNA origami. Binding to DNA causes a slight red-shift of the absorption spectrum and an overall decrease of ϵ in the visible wavelength region. Additionally, the absorption peak associated with the DOX-Mg^{2+} complex at *ca.* 570 nm disappears, when the stronger DOX-DNA interaction causes dissociation of the weakly bound DOX-Mg^{2+} complexes. The DOX fluorescence quantum yield (Φ) decreases upon DNA addition, as shown in the middle panel of Figure 3b for excitation wavelength 494 nm. The fluorescence spectra were corrected for the decrease of ϵ_{494}, which also leads to decreasing fluorescence intensity when less light is absorbed in the sample. By the end of the titration and at a base pair/DOX molar ratio of approximately 15, the titration is close to a saturation point with ϵ_{494} decreased by *ca.* 35%, and Φ *ca.* 89% (Figure 3b, right panel). The absorption and fluorescence spectra of bowtie, double-L, capsule, and 24HB DNA origami appear highly similar to the triangle DNA origami, and are presented in the Supplementary Figure 4.

Increasing the amount of DNA origami in the sample causes a discernible scattering effect, which is stronger for the 3D DNA origami shapes than for the 2D shapes (Supplementary Figure 10). In the absorbance measurement, this is observed as a slight elevation in the spectrum baseline during the titration, which has an influence on the apparent sample absorbance. While the scattering effect is minor compared to the spectral changes of DOX, it leads to moderately increased apparent ϵ of DOX in the end of the titration for the 3D shapes (Supplementary Figure 5). For this reason, further analysis of the binding was based on the fluorescence data, which is less affected by the light scattering effects. Analysis of the absorption data was then carried out using the parameters obtained from the analysis of the fluorescence data.

Interpretation of the experimental results through a molecular binding model. DOX has been proposed to bind dsDNA through two prevalent mechanisms: intercalation between G–C base pairs, and binding at A–T rich areas (47) *via* minor groove binding through electrostatic interactions, which is also considered an intermediate step in the intercalation process (46). The fluorescence of DOX has been shown to be fully quenched in the more strongly bound DOX-GC complex (intercalation), while the weaker DOX-AT complex remains gently fluorescent in double-stranded DNA (dsDNA) (47, 62). This is also fully supported by our observations; when DOX electrostatically binds to a single-stranded DNA (ssDNA), the DOX fluorescence does not quench, although its absorption spectrum changes in a similar fashion as in the case of DNA origami (Supplementary Figure 8). For describing the observed decrease of Φ and ϵ when increasing the concentration of DNA origami, we thus applied a 1:2 molecular binding model for including both modes of interaction and the formation of two distinct DOX-DNA complexes with different association constants (K_{11} and K_{12}) and fluorescence quantum yields (Φ_{11} and Φ_{12}).

In the right panel of Figure 3b, the dependence of Φ and ϵ_{494} on the base pair concentration in the triangle DNA origami sample is described by a fit according to Equations (2) and (4) [Equations (1–7) can be found in the experimental section of the Supporting Information]. The model suggests that in the fluorescence measurement, the two DOX-DNA complexes with an average K_{11} = $(2.0 \pm 0.3) \times 10^5$ M^{-1} and K_{12} = $(2.6 \pm 0.2) \times 10^5$ M^{-1} can be differentiated from

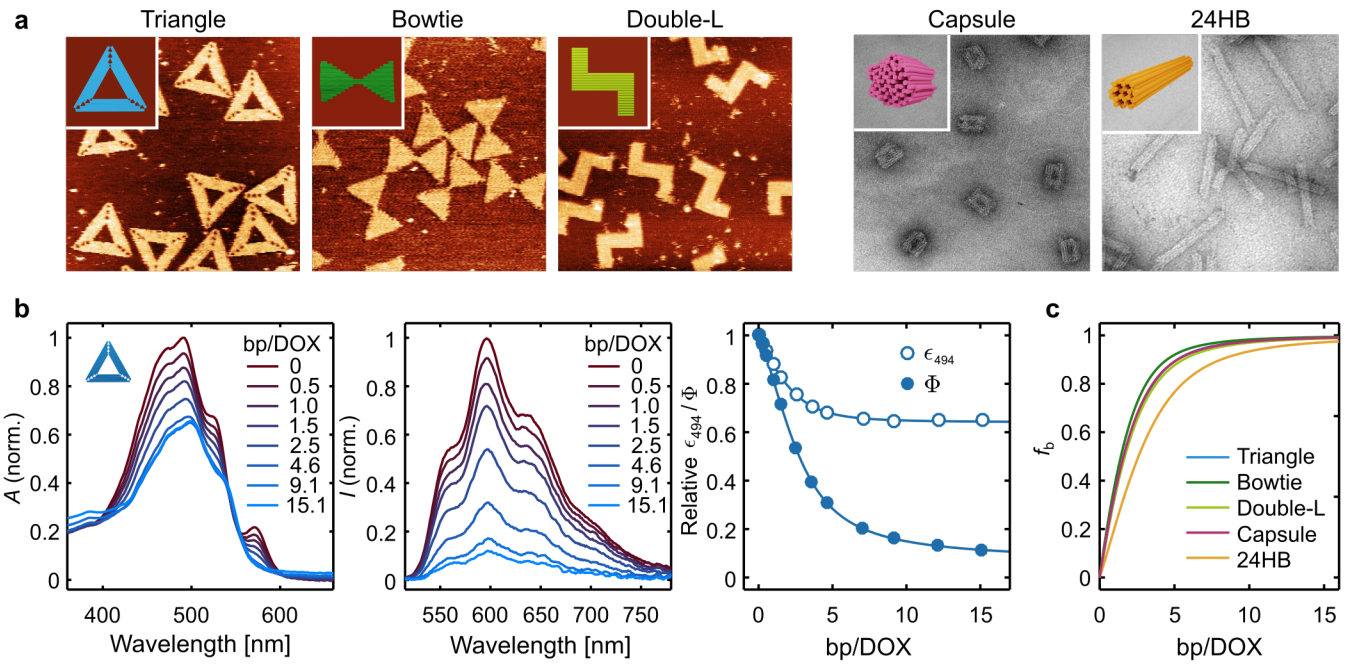

Figure 3. DNA origami titration experiments for determining the DOX-loading properties of different DNA origami structures. **a** The models and microscopy images of the studied 2D and 3D DNA origami. The triangle, bowtie, and double-L 2D DNA origami are shown on the left accompanied by atomic force microscopy (AFM) images. The 3D DNA origami – the capsule and the 24-helix bundle (24HB) are shown on the right in TEM images. The AFM images are 500 nm × 500 nm in size, and the TEM images are 300 nm × 300 nm. **b** Representative changes in the absorption spectrum (left panel) and fluorescence emission after 494 nm excitation (middle panel) of 3 μM DOX when the concentration of DNA base pairs (bp) in the solution is increased. The spectra have been measured for the triangle DNA origami after the system has reached equilibrium. The DNA concentration at each titration step is expressed as the molar ratio between DNA base pairs and DOX molecules in the sample (bp/DOX), and indicated in the legend. The fluorescence spectra in the middle panel have been corrected for the decrease of the molar extinction coefficient at the excitation wavelength (ϵ_{494}), and represent the quantum yield of the emitting molecules. The relative decrease of ϵ_{494} and Φ during titration with the triangle DNA origami are shown against the amount of DNA added (bp/DOX ratio) in the right panel. The measured values for ϵ_{494} and Φ have been fitted with a 2-component binding model as described in the text. The corresponding spectra and titration isotherms for the other DNA origami structures are presented in the Supplementary Figure 4. **c** Increase of the fraction of bound DOX molecules (f_b) when the DNA base pair concentration in the sample increases, obtained by fitting the fluorescence data.

each other by the extent of fluorescence quenching ($\Phi_{11}/\Phi_0 = 0.52 \pm 0.07$ and $\Phi_{12}/\Phi_0 = 0.067 \pm 0.009$), but in terms of light absorbance, their physical properties are similar ($\epsilon_{11}/\epsilon_0 = 0.58 \pm 0.08$ and $\epsilon_{12}/\epsilon_0 = 0.67 \pm 0.09$ for 494 nm).

The binding model can thus be seen to present a reasonable approximation for the behavior of the system and the changes of the physical properties of DOX (Φ and ϵ) upon DNA addition by taking into account the two types of binding modes, and essentially, their distinct fluorescence properties. The determined values of K_{11} and K_{12} are in the same range and order of magnitude as in previous studies (47, 62) – nevertheless, we note that generalization of the fitting results and the obtained parameters outside the presented experimental conditions should be carried out with caution due to the simplifications of the model. A comparison of the fitting parameters for all DNA origami structures presented in this study can be found in the Supplementary Table 2.

The fraction of bound DOX molecules at each bp concentration can then be obtained from the fit according to Equation (3), which enables a comparison of the DOX loading properties for different kinds of DNA origami shapes (Figure 3c). It appears that the DNA origami superstructure has relatively little effect on how much DOX can be bound to the structures, as all curves in Figure 3c are rather similar. In the beginning of the titration, the fraction of bound DOX increases sharply when DNA is introduced into the sample. The maximum number of bound DOX molecules per base pair is reached at 0.36 ± 0.10 (Supplementary Figure 6).

DOX release upon nuclease degradation

After determining that all the studied DNA origami shapes have rather similar DOX loading capacity, we studied their superstructure-related differences towards DNase I digestion. When the DOX-loaded DNA origami structures are digested by DNase I, the dsDNA structure of the DNA origami breaks down as the nuclease cleaves the DNA into short ssDNA fragments. As the ϵ_{260}/nt for ssDNA is higher than the ϵ_{260}/nt for dsDNA (63), the process can be followed from the increase of the A_{260} of the sample. The bound DOX is released when the double-helical DNA structure unravels, observed as a recovery of DOX fluorescence. In order to follow both processes in detail, we employed a simultaneous kinetic spectroscopic characterization of both the increase of DNA absorbance and the recovery of DOX fluorescence during DNase I digestion in DOX-loaded DNA origami samples.

We also studied the effect of the DOX loading content, which can be tuned with the DOX concentration during loading: at 3 μM DOX (molar ratio bp/DOX = 4.5 ± 1.5), the average density of DOX bound in the structures was determined as 0.20 ± 0.05 bound DOX molecules/bp, based on the measured DOX fluorescence quenching and parameters obtained for the thermodynamic DOX-DNA binding model. Doubling

the DOX concentration to 6 μM (bp/DOX = 2.3 ± 0.8), while keeping the concentration of DNA base pairs constant, leads to a slightly higher number of bound DOX molecules; 0.33 ± 0.07 DOX/bp. It is noteworthy to acknowledge that free, unbound DOX in the solution was not removed before the digestion experiments, as this would disturb the binding equilibrium and promote an additional pathway of DOX release during the incubation. A comprehensive list of the bp/DOX ratios and amounts of loaded and free DOX in the samples can be found in the Supplementary Table 4.

When the DNA origami are digested by DNase I, the A_{260} of the samples increases roughly linearly, until reaching a saturation point where the structures are fully digested. Full digestion of the double-helices increases the A_{260} of the 2D structures on average by (30 ± 2)%, and the A_{260} of the 3D structures by (22 ± 4)%. Theoretical values predicted by calculating the ϵ_{260}/nt for intact and digested structures according to Equation (1) are (46 ± 3)% and (44 ± 5)% for 2D and 3D structures, respectively. The smaller measured values in comparison with the predicted values suggest the initial ϵ_{260}/nt values to be slightly overestimated for all DNA origami structures, and more so for 3D structures that cause more light scattering in the intact state (see Supplementary Figure 10).

Following the A_{260} increase reveals that the dsDNA structures of the DNA origami break down at distinct rates depending both on the DNA origami superstructure and the DOX concentration in the sample (Figure 4a). In general, the 3D shapes are digested more slowly than the 2D structures, and their digestion profiles are thus presented over 41 hour incubation, while a shorter (< 1 h) incubation is sufficient for studying the DNase I digestion of the 2D structures. The fastest digestion is observed for the triangle DNA origami in the absence of DOX, with the structures being completely degraded within 20 minutes of incubation in the presence of 34 U/mL DNase I. Notably, increasing amounts of DOX slow down the DNase I digestion by a considerable extent. The digestion rates of all structures are compared in Figure 4d, where the effect of slightly different DNase I concentrations for the 2D and 3D structures has been taken into account by normalizing the rates per unit of DNase I.

To further support the spectroscopic results of the DNA origami digestion, an agarose gel electrophoresis (AGE) analysis was carried out parallel to the kinetic DNA absorption and DOX fluorescence measurements (Figure 4b). Incubating the 24HB and the capsule with 28 U/mL DNase I leads to a lower band intensity and an increased electrophoretic mobility of the samples, indicating a reduced size of the partly digested DNA origami. In the case of complete digestion, the bands disappear completely. The observed effects of DNase I digestion in AGE after 17 h and 41 h are well in agreement with the structural digestion determined from the A_{260} increase in Figure 4a. Likewise, incubating the 2D structures for 17 h in the same conditions as for the 3D structures leads to complete digestion of the structures, observed both as a stabilized A_{260} signal and a disappearance of the DNA origami bands in AGE (Supplementary Figure 11).

When DOX-loaded DNA origami are digested by DNase I, DOX release is observed as a recovery of DOX fluorescence. Figure 4a presents the fraction of initially loaded DOX molecules released from the DNA origami during the DNase I incubation. For both DOX concentrations, the fractions of bound and released DOX are closely linked to the fraction of intact dsDNA residues in the samples. As a result, the total amount of loaded DOX is released more slowly from the 6 μM DOX samples than from the 3 μM DOX samples. The cross-correlation between released DOX and the intactness of the DNA origami structures for the 3 μM DOX samples is shown in Figure 4c.

While the 6 μM DOX samples are digested more slowly, they also contain a higher amount of DOX than the less densely loaded 3 μM DOX samples. When the loading density of the structures is taken into account and the DOX release is considered in terms of the absolute number of released DOX molecules, the difference between the DOX release properties of 3 μM and 6 μM DOX samples decreases. In Figure 4e, the DOX release rates are compared in terms of the number of DOX molecules released per unit of time per unit of DNase I. A general trend observed for the different structures is that while the 6 μM samples are able to release more DOX into the solution, the inhibition of DNase I function is still a stronger effect and leads to faster drug release rates from the 3 μM samples.

Discussion

DOX loading and choice of conditions

In common experimentation with DNA origami, the buffer of choice is typically a Tris-HCl or a TAE buffer, either supplemented with on average 10–20 mM Mg^{2+}. These conditions have been generally found to be appropriate for stabilizing the DNA origami structures: the divalent cations effectively screen the electrostatic repulsion between the negative charges of closely packed phosphate backbones, and the typical pH at 8.0–8.3 is in the optimal buffering range of Tris-based buffers. As it is important to retain the structural integrity of DNA origami nanostructures throughout experimental procedures, these conditions are also commonly used together with DOX – particularly during incorporating (loading) the DOX into the DNA origami structures. Still, the question of whether these conditions can cause unexpected or undesired behavior of DOX, or change its spectroscopic properties in terms of ϵ or Φ in a way that can lead to a misinterpretation of spectroscopic observables, has been left almost entirely unaddressed.

In our spectroscopic analysis, we found that if the pH is at or above 8.0 and the $MgCl_2$ concentration is at mM range, the environment will lead to DOX heterogeneity either in terms of charge (deprotonation) or formation of DOX-Mg^{2+} complexes (Figure 2a-c). In general, the spectroscopic properties of DOX are obviously associated with the prevalent solution pH, but unfortunately this fact is often brushed aside (34, 40). For example, it has been stated that the amount of DOX released from the DNA structures increases with decreasing pH (34), but our results strongly suggest that the observed ele-

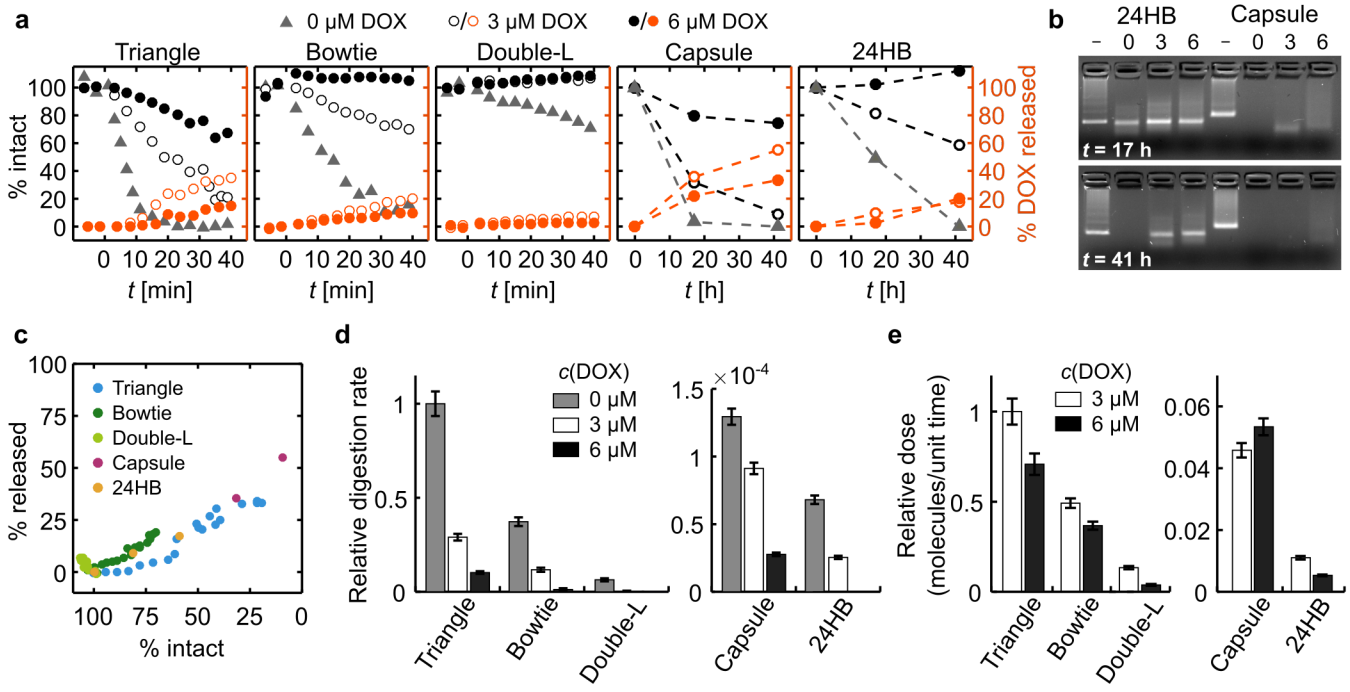

Figure 4. Digestion of the DNA origami nanostructures and DOX release during incubation with DNase I. **a** Comparison of the degradation of the dsDNA framework of the DNA origami shapes and the resulting DOX release. The structural integrity of the DNA origami (% intact), as determined from the increase of the A_{260} signal, is shown with the gray triangle markers for the samples without DOX, and with the empty and filled black markers for the samples containing DOX at either 3 or 6 μM concentration. For the samples with 3 and 6 μM DOX, the orange markers depict the fraction of released DOX molecules relative to the initial concentration of bound DOX molecules. **b** Agarose gel electrophoresis (AGE) analysis of the 24HB and capsule DNA origami samples after 17 h and 41 h digestion. The first lane for each sample (-) contains the DNA origami without DNase I or DOX; the lanes marked with 0, 3, and 6 contain the indicated concentration of DOX in μM, with additional 28.2 U/mL of DNase I in each sample. **c** Cross-correlation plots of % DOX release *vs.* % intact for all DNA origami shapes at 3 μM DOX. **d** DNase I digestion rates relative to the rates measured for the triangle DNA origami. DNase I digestion rate denotes the decrease rate of the relative fraction of intact dsDNA structures. **e** Relative dose stands for the number of DOX molecules released per unit of time and takes into account the absolute concentration of DOX molecules loaded into the DNA origami in the beginning of the incubation. Both the digestion and release rates have been normalized to the concentration of DNase I in the samples (34 U/ml for the triangle, bowtie, and double-L, and 28 U/mL for the 24HB and the capsule), and further shown relative to the rates measured for the triangle DNA origami.

vation in DOX absorbance and fluorescence may just arise from the spectroscopic properties of DOX (high absorbance and emission at low pH) and not from the DOX concentration change.

While the observations are important for validating the spectroscopic results obtained at these buffer conditions, they also reflect the aggregation behavior of DOX observed in different Tris-based buffers supplemented with $MgCl_2$ (Figure 2d). The precipitation of DOX during storage at RT indicates two major aggregation pathways in line with the previous literature (47, 50, 58, 59). The more prevalent one is observed at pH $\geq$ 8.0 when the DOX concentration is above the solubility of the deprotonated molecules (0.55 mmol/L) (50). The second mechanism is linked to the complexation with Mg^{2+}, which causes slight DOX aggregation and precipitation regardless of the DOX concentration (from μM to mM range). This aggregation pathways becomes notable at lower DOX concentrations where the magnesium:DOX ratio is high and the contribution of the more significant aggregation mechanisms is smaller.

The experimental conditions and the protocol of plain DOX incubation used here are consistent with the commonly used strategies to load DOX into DNA nanostructures (34, 36, 44). Incubation of DOX alone at millimolar concentrations and at pH 8.0 leads to an almost identical precipitate as using the reported DOX-loading approaches. Therefore, it is fairly reasonable to assume that the method for preparing purified DOX-loaded DNA origami structures *via* the same precipitation protocol (34, 36, 44) can likewise result in samples containing highly aggregated DOX.

Our results show that typical DNA origami buffers supplemented with $MgCl_2$ are not suited for unequivocal DOX experimentation. While the pH of the buffers can be readily adjusted to pH < 8.0, magnesium may be more challenging to exclude as it is a crucial counterion for screening the charged phosphate groups. While DNA origami have been shown to remain stable in low-Mg^{2+} conditions (55), addition of positively charged reagents such as DOX can be expected to influence the number of Mg^{2+} ions associated with the backbone in low-Mg^{2+} buffers, and thus may compromise the structural stability.

In summary, these findings may be used as a cautionary tale on how to choose the conditions for DOX loading. Certain compromises are inevitable and required for fitting together DNA origami and DOX, which both have a different optimal environment. According to our results, it is important to acknowledge the effect of these concessions in the loading process as well as in long-term drug release and storage.

DOX loading – efficiency and features

When DNA origami structures are mixed with DOX, the high DNA-binding affinity of the drug leads to a fast formation of non-covalently bound DNA origami-drug complexes. In our titration experiments, we studied and compared the DOX-loading capacities of five distinct DNA origami structures. In our experimental conditions and in the loading experiments presented in Figure 3, the maximum DOX loading content was determined to be 0.36 $\pm$ 0.10 DOX molecules bound per one DNA base pair for all of the tested structures. The value is in line with a number of previous studies for calf thymus dsDNA, where the maximum binding efficiency of DOX had been determined to be in the range of $\sim$0.29–0.37 DOX molecules per base pair (47, 64).

The association of DOX with DNA has been shown to take place predominantly *via* intercalation and minor-groove binding. As intercalation is known to affect the DNA helicity and cause torsional twist in DNA origami structures (35, 65), we further elucidated the intercalation effect with the double-L structure and an atomic force microscopy (AFM) -based characterization. AFM images of DOX-loaded double-L DNA origami (see Supplementary Figure 7) show that although the double-L is designed to be in a *trans*-form (arms on the opposite sides of the middle bar), the DOX binding can twist it to a *cis*-form (arms on the same side of the middle bar) in a concentration-dependent manner. Importantly, all the structures remain intact upon DOX addition. Our results also strongly support the presence of a minor-groove binding mode that leads to a lesser extent of DOX fluorescence quenching (47, 62). It is thus highly likely that both modes of binding contribute to the loading capacity of the DNA origami structures in our experimental conditions.

Interestingly, roughly identical amounts of DOX were incorporated into all of the tested DNA origami shapes in terms of density of the drug molecules on the DNA – the DOX loading capacity of the DNA origami being thus only affected by the size of the structures in base pairs, and not by other design-related features. This is a rather surprising observation, as the steric hindrance from the compact arrangement of DNA helices could be expected to lead to a restricted accessibility of DNA helices and intercalation sites on the DNA origami, and particularly so for the 3D structures. In fact, such kind of restricted loading has recently been observed for the bis-intercalator YOYO-1 (49). The different behavior we observe for DOX might arise from the different binding mechanisms of the two drugs. Some subtle differences between the studied structures might have also been left undetected by our analysis methods.

Although our observations of low DOX-loading capacity of DNA origami are in agreement with the earlier dsDNA data, they seem to contradict many previous studies on DNA origami-based DOX delivery, where the reported concentrations of bound drug molecules in DNA origami structures are often higher than the concentration of DNA base pairs in the sample. For instance, the amount of DOX bound by the triangle DNA origami has been reported to range from 2.8–19 DOX molecules per base pair (37, 39, 41) to as high as 55 DOX molecules per base pair (34, 36).

While it is rather obvious that intercalation cannot be the only DOX binding mechanism behind the reported high DOX loading contents, the other possible processes, such as minor-groove binding or even external aggregate formation through stacking interactions (47), are rarely discussed. Our results support the interpretation that all three mechanisms might play a role in the loading process depending on the choice of experimental conditions, with DOX aggregation presenting a plausible explanation for DOX loading contents well above a loading density of DOX/bp > 1. Indicated *e.g.* by the previously reported long loading times and strategies, as well as by the kinetics in both *in vitro* and *in vivo* experimentation, the observed therapeutic effects could likewise be linked to DOX aggregates with only little or no contribution from the well-formed DOX-DNA origami complexes (34, 36, 37, 41, 42). As a result, the actual mechanism of anticancer action and therapeutic efficacy of these DOX-DNA origami samples without any DOX-loading optimization remains still unknown.

DNase I digestion leads to DOX release at superstructure-dependent rates

As an important prerequisite for any biomedical application, we simulated the possible degradation pathways of the complexes in DNase I-rich environments. DNase I was selected as a degradation agent as it is the most important and active nuclease in blood serum and mammalian cells. In addition, DNA nanostructure digestion by DNase I have been previously studied (52, 54, 66–69), but not with this kind of approach that allows monitoring of DNA origami cleavage and drug release simultaneously.

By following the change of DNA absorbance at 260 nm upon DNase I addition, it is possible to resolve superstructure-dependent DNA origami degradation rates. The stability varies from structure to structure, which has also been observed in the previous studies. In general, DNA origami structures are known to be more resistant toward DNase I digestion than plain dsDNA, and it seems that the rigid, closely packed helices and the strand routing play a role in the achieved resiliency (52, 54, 66, 68, 69). DNase I is a strong minor-groove binder (70). Its mechanism of action induces widening of the minor groove and a bending toward the major groove, thus making the flexible regions of DNA molecules and structures more susceptible towards cleavage (52, 70). Along with the increasing compactness and rigidity of DNA origami, the possible cleavage sites become less accessible (53). Recently, it was also found out that the stability is DNA crossover-dependent (69).

The superstructure-dependent DNA origami digestion is notably slower for 3D than 2D structures; in the most extreme case (triangle *vs.* 24HB), by roughly four orders of magnitude (Figure 4a and 4d). However, the percentage of released DOX correlates well with the degradation level of the DNA origami (Figure 4c), thus enabling customized drug release over a rather wide time window. The plain 2D structures follow similar digestion profiles as reported earlier

(52), and the increased stability of the 24HB compared to the capsule may originate from design features such as a more compact and regular structure and higher amount of scaffold crossovers. However, here it is noteworthy that when the structures are loaded with DOX, the digestion slows down and adds one more controllable parameter to the tunable release profile. The underlying mechanism is most likely DOX-induced DNA protection, which has also been previously observed for dsDNA with variable sequences (71). In other words, the bound DOX partially interferes with the minor-groove binding of DNase I.

Furthermore, to achieve reasonable time scales for the digestion rate comparison, we have here applied a DNase I concentration ($\sim$30 U/mL) that is remarkably higher than for example in blood plasma (0.36 ± 0.20 U/mL (72)). Obviously, DNase I concentration is not the only factor that affects the digestion rate, as the DNase I activity is also dependent *e.g.* on the buffer, salt concentration and temperature. However, as the concentration of DNase is essentially defined through its activity, the acquired results set an appropriate starting point to estimate the susceptibility and the drug release capacity of distinct DNA shapes. For that, the calculated relative dose (released amount of DOX / unit time) for each structure serves as a feasible measure of the actual release efficacy (Figure 4e). Interestingly, 6 μM DOX loading yields lower doses than 3 μM, however, in this setting the shape/structure of DNA origami plays a more crucial role. In a nutshell, the distinct DNA shapes used in this work and the applied DOX-loading levels together provide a broad selection of relative doses for fully engineered DOX delivery (Figure 4e).

Conclusions

We have shown that the release of the common therapeutic drug DOX from DNA nanostructures upon DNase I digestion can be customized by rationally designing DNA superstructures and adjusting the concentration of DOX. Importantly, our spectroscopic analysis of DOX-loaded DNA origami and free DOX under different conditions reveals that a number of studies have poorly estimated DOX loading capacity and overlooked the effect of DOX self-aggregation, which both may lead to suboptimal loading, questionable outcomes and misleading interpretation of the actual drug efficacy. Therefore, our results may also help in explaining previous, often incoherent reports on DNA origami-mediated DOX delivery.

Our results show that both the superstructure and rigidity of DNA origami have an impact on its stability against nucleases, which is in agreement with previous studies (52, 54, 66, 68). The stiffness and resilience of DNA origami achieved by the close packing of helices may, on the other hand, deteriorate the loading capacity of DNA-binding drugs (49). Nevertheless, here we observed nearly identical DOX loading properties for all DNA origami shapes, but drastically different digestion and release profiles. Interestingly, increasing the amount of loaded DOX slows down the digestion, which is plausibly associated with restricted DNase I cleavage due to the interfering DNA-bound DOX.

All of our observations underline the significant potential of DNA origami structures in drug delivery applications. Here we employed plain DNA origami without further modifications, but by taking advantage of their unsurpassable addressibility and modularity, multifunctionalities can be further realized. In the bigger picture, we believe our findings will help in building a solid ground for the development of safe and more efficient DNA nanostructure-based therapeutics with promising programmable features.

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Author contributions
H.I. designed and performed the experiments, modeled the data and wrote the manuscript. B.S. and A.H.-J. designed and performed the experiments. A.K. conceived the project and designed the experiments. M.A.K., T.L. and J.A.I. supervised the work and designed the experiments. V.L. conceived and supervised the project, designed the experiments and wrote the manuscript. All authors analyzed and discussed the data and also commented on and edited the manuscript.

Additional information
Supplementary Information accompanies this paper.

Competing interests
The authors declare no competing interests.

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