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Prevention of doxorubicin sorptive losses in drug delivery studies using polyethylene glycol

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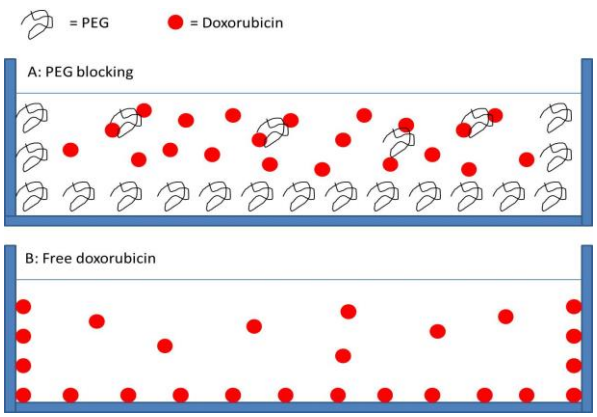
The nonspecific sorption of hydrophobic pharmaceuticals on reaction vessel surfaces,raises serious analytical challenges for their accurate quantification. Systematic error due to sorptive loss of analytes may result in significant overestimation of drug loading on nanomaterial-based drug delivery systems (DDS), leading to inaccurate determinations of dosage and DDS efficiency. We evaluated sorptive losses of doxorubicin (DOX), an effective chemotherapeutic, in polystyrene based 96-well plates, and proposed a simple but effective method to prevent the nonspecific sorption of DOX using trace concentrations of polyethylene glycol (PEG). Relative to widely used proteinaceous and surfactant surface blocking agents, PEG is effective, easy to use, and does not interfere with drug loading to the DDS.

Introduction

Doxorubicin (DOX) is widely employed as an effective chemotherapeutic for treatment of various solid tumors, but is associated with significant adverse side effects including cardiomyopathy, potentially resulting in congestive heart failure.[1-2] To address this issue, research into drug delivery systems (DDSs) that use nanomaterial-based drug carriers designed with targeting functionality could enhance DOX effectiveness by delivering high drug concentrations directly to cancerous tissues. Such a DDS would decrease both required concentrations, and by being targeted, reduce adverse side effects in blood and other organs.[3-7] Accurate quantification of drug loading capacity to nanomaterials is not only critical to evaluate the efficacy of the nano-drug carriers in DDS development research, but also important to determine the dosage of the DDS for clinical trials. However, quantitative evaluations of DDS efficacy have been significantly hampered by the non-specific sorption of DOX to various plastic containers during storage and analysis[8-10], with photo-degradation further deteriorating data quality.[11-13] Gold nanoparticles (AuNPs) are an example of nano-drug carriers for DDS development. When evaluating the loading capacity of DOX onto AuNPs, there are three experimental steps: drug loading to AuNPs, separation of free DOX from bound fractions (*i.e.*, DOX-AuNP conjugates), and instrumental quantification of the free fraction. The loading capacity of DOX to each AuNP is measured by the optical signal difference (absorbance or fluorescence) before and after DOX loads to the nanoparticles, as shown in Eq. 1.



Scheme 1. Schematic representation of prevention of non-specific sorptive losses of DOX in plastic reaction vessel surfaces by PEG.

$$N_{Loading} = (C_{total} - C_{free}) / C_{AuNP} \qquad (1)$$

Where $N_{Loading}$ is the number of DOX adsorbed to each AuNP, C_{total} and C_{free} are the total and free concentration of DOX, and C_{AuNP} is the concentration of AuNPs in the drug loading system. Any DOX adsorbed to the container surfaces (*e.g.*, microcentrifuge tubes, micropipette tips, or 96 well plates), is attributed by Eq.1 to the loading capacity of AuNPs for DOX, which can overestimate the loading capacity of the drug carriers. The assumption underlying such calculations is a negligible sorptive loss of DOX, which in reality has been disproven in several studies. [8-10] To date, the adsorption of DOX to various material surfaces including glass, siliconized glass, polyethylene, polypropylene, polytetrafluoroethylene, polyvinylchloride, and cellulose dialysis membranes has been

well documented.[8-10] However, there is no data for the adsorption of DOX on polystyrene 96-well plates, which are routinely used for laboratory fluorescence quantitation. Systematic non-specific sorption experimental error, if present, would seriously affect fluorescence measurements, leading to false conclusions. Further, there is little research on approaches to prevent DOX sorptive losses and ensure analytical accuracy, despite the drug's widespread use in this context.[14-16] To address these problems, we propose a simple but effective method to prevent sorptive losses by incorporating trace (part-per-million) concentrations of polyethylene glycol (PEG) into the buffer used to dissolve DOX (shown in the scheme 1). Critically, the addition of PEG does not interfere with the loading of DOX to nanoparticle surfaces, thereby ensuring accurate quantification of drug loading capacity.

Results and discussion

Sorptive losses of DOX

The sorptive loss of DOX onto various experimental plastic containers might seriously compromise quantitative evaluations of Drug Delivery System (DDS) performance.[8-10] For example, if loading 10 μM DOX to gold nanoparticles (AuNPs), the DOX concentration is normally 10^2-10^5 times that of the concentration of the nanomaterials for experimental use (for example, the concentration of widely used 13 nm AuNPs is about 10 nM). Under these conditions, 20-50% (2-5 μM) cumulative sorptive losses could be expected during multi-step-experiments (including drug loading, centrifugation in polypropylene microcentrifuge tubes, and during quantification in 96-well plates). Consequently, the total available concentration of DOX in the solution would be 50-80% (*i.e.,* 5-8 μM) of the originally added concentrations. If we assume the real loading capacity is 500 DOX/AuNP, the calculated loading capacity would be 700-1000 DOX/AuNP, which would be 40-100% overestimated; such overestimates result in unreliable conclusions.

The non-specific sorption of DOX to polypropylene microcentrifuge tubes was evaluated as these tubes are among the most frequently used plastic vessels in laboratory experiments. The adsorption of DOX (7.5 μM DOX in 100 μL 5 mM HEPES buffer, pH 7.6) onto polypropylene tube surfaces decreased fluorescent signal intensities ~ 20% over 30 min; lower DOX concentrations (2.5 μM) experienced relatively greater sorptive losses of 40% over 10 min (Fig. 1A), which is consistent with previously published data.[8] To elucidate the mechanism of DOX adsorption, the influence of NaCl, ethanol, and ethylene glycol on the amount of DOX adsorbed to the tube was evaluated. Adsorption would be essentially attributable to electrostatic interactions if the addition of NaCl decreased adsorption due to the charge screening effect, while adsorption would be primarily driven by hydrophobic interactions if the presence of organic solvents (EtOH and EG) decreased sorptive losses of DOX. Sorptive DOX losses were in fact unaffected by NaCl concentrations (Fig. 1A) but reduced by ethanol and ethylene glycol (EG, as discussed later) indicating DOX adsorption to container surfaces is governed by hydrophobic rather than electrostatic interactions.

We next evaluated DOX sorptive losses in polystyrene 96-well plates, which are again widely used for fluorescence-based DOX quantification. DOX loading capacity, as observed by Langmuir isotherm, was determined as 520 nM per well on 96-well plates (Fig. 1C). At 520 nM, assuming the size of each DOX molecule is about 3.17 nm^2 with a diameter of 1 nm, (based on the theoretical estimation of DOX molecule size of 1.026 nm in diameter using the Global Minimum approach at the lowest energy) we can determine each 100 μL well working volume can sorb 3 X 10^{13} DOX molecules on each 95 mm^2 useable well surface. The Langmuir isotherm and aforementioned calculations support a monolayer adsorption model. The adsorption of DOX to plate well surfaces is a relatively fast process with the adsorption-desorption equilibrium achieved within 40 min (Fig. 1B), a typical time interval for drug adsorption experiments with nanomaterials.[17-19]

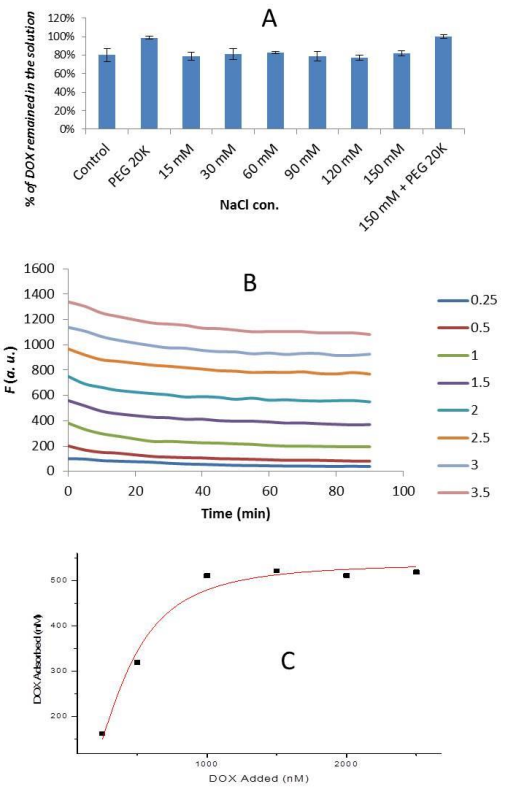


Figure 1. Sorptive losses of doxorubicin to polypropylene micro-centrifuge tubes (A) and polystyrene 96-well plates (B and C). A: The effect of salt and/or PEG 20K on DOX sorption onto microcentrifuge tube surfaces. In the control tube, no chemicals other than DOX aqueous solution were added. B: Decrease in the fluorescence of various DOX concentrations (from 0.25 to 3.5 μM) within plate wells over time; C: The Langmuir isotherm for non-specific sorption to plate-well surfaces.

PEG effect on sorptive loss of DOX

Currently, there are several established agents used for surface blocking including BSA and the surfactants Tween and Triton X-100, which are efficacious in preventing sorptive losses in various bioassays.[20] However, these traditional blocking methods have limitations such as additional and time-consuming plate treatment steps (*e.g.*, 0.5-12 h for BSA blocking), generating air bubbles in the sample solution during mixing, and potential for interference with DOX loading onto nanomaterials. All these technical issues can be overcome by using low concentrations of PEG as the blocking agent. The presence of 10 ppm of PEG 20K in the plate wells produces neither air bubbles nor inhibition of DOX loading, as evidenced by retention (of around 100%) of DOX fluorescence intensity (Fig. 2). Initially, we tested the effects of PEG molecular weight (MW) and concentration on DOX adsorption to the surfaces of polystyrene plate wells (Fig. 2 and Fig. S1). This trial demonstrated that although PEG 1000 is helpful (~ 98% of DOX was retained, only 2% sorptive loss), PEG of larger MW were more effective. For example, only 10 ppm of PEG 20K (0.5 μM) demonstrated comparable blocking efficacy as 100 ppm of PEG 4K or 1000 ppm of PEG 1K (Figs. 2 and S1). Consequently, the role of PEG MW and concentration on sorptive loss of DOX was systematically evaluated (Fig. S1), producing the conclusion that 10 ppm of PEG 8000 is generally effective in addressing sorptive DOX losses in 96 well plates. Similar blocking protection for DOX in the presence of 10 ppm of PEG 20K within polypropylene microcentrifuge tubes was observed (Fig. 1A), indicating the universality of PEG blocking in plastic vessels, which is consistent with results demonstrated in homogeneous biosensor development.[20]

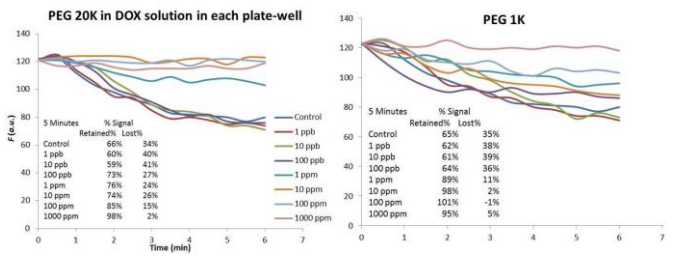


Fig. 2. The effects of PEG (various concentrations and molecular weights) on DOX adsorption onto 96 well plate surfaces. Inset tables demonstrate PEG protection is a function of both molecular weight and concentration. In the control wells, no chemicals other than DOX aqueous solution were added.

There are two potential mechanisms contributing to PEGs' reduction of sorptive losses of hydrophobic drugs such as DOX from the drug loading solution (Scheme 1). First, PEG molecules, especially those of higher molecular weight (*i.e.*, ≥ 4K), can adsorb to plate surfaces, preventing DOX adsorption as evidenced by the Langmuir adsorption isotherm (Fig. 1C)[20-22]. The second possible mechanism is that the addition of PEG decreases the aqueous buffer polarity, thereby increasing DOX solubility, as observed and utilized in organic synthesis by other groups.[23-25] If PEG was in fact modifying the polarity of the solution, we would expect molecules with similar properties to exert similar effects. Our results with ethanol and EG also support this mechanism, as 10% ethanol or EG significantly alleviated sorptive losses of DOX relative to a control lacking ethanol or EG, albeit less effective than 10 ppm of Tween 40, Triton X-100, or PEG 20K (Fig 3).

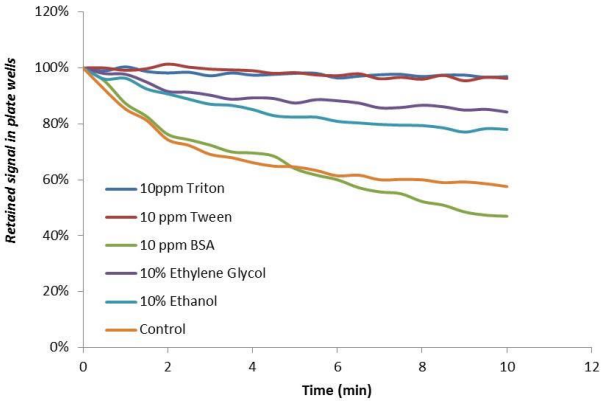


Figure 3. Comparison of several surface-blocking agents with 10% (*v/v*) ethylene glycol and ethanol. In the control wells, no chemicals other than DOX aqueous solution were added.

Additionally, it is also possible DOX could bind to PEG molecules, reducing DOX molecule polarity and thereby increasing solubility. We tested this potential mechanism by studying if DOX was adsorbed to PEG-coated AuNPs (pre-functionalized with thiolated PEG 2K). Since AuNP is an excellent fluorescence quencher (based on nanoparticle surface-energy transfer (NSET), it would quench the fluorescence of DOX upon binding.[26-28] However, after DOX solution was added to the PEG-SH-AuNP solution and incubated under room-temperature for 30 min, no DOX adsorption (as evidenced by fluorescence quenching) was observed by monitoring the fluorescence intensity of the supernatants. All DOX signals were recovered by centrifugation, demonstrating DOX does not adsorb to PEG (data not shown).

PEG effect on DOX loading on gold nanoparticles.

As mentioned previously, many surface blocking agents, when employed to prevent sorptive losses of DOX to plastic vessel surfaces, may also block the surface of the drug carrier (*i.e.*, gold nanoparticles in the current work), significantly affecting drug-loading capacity. We compared several surface blocking reagents (*i.e.*, BSA, Triton X-100, Tween 20, and PEG 20K) for their capacity to prevent sorption of DOX to polystyrene plate wells (as shown in Fig. 3) but also their interference of DOX loading to AuNPs. DOX solution fluorescence in plate wells was recorded over 10 min in the presence of these various blocking reagents (10 ppm) with AuNPs added into each well after 240 s. Over the first 240 s, there was a 19% decrease in DOX fluorescence (as indicated by the distance *a*) for the control sample (no blocking reagent) in the plate wells; in contrast, Triton X-100, Tween and PEG 20K maintained a near constant fluorescence level, indicating they effectively prevented sorptive losses of DOX to plate well surfaces (Fig. 4). However, 10 ppm BSA did not prevent the DOX adsorption

to the plate surface, which is reasonable considering the hydrodynamic diameter of BSA (~7 nm) and the high protein binding affinity of DOX (~75%). Presumably, DOX adsorbed to plate well surfaces between adjacent BSA molecules, and/or to BSA itself forming BSA-DOX conjugates. Interference of DOX loading to AuNP surfaces was evaluated by comparing fluorescence quenching after addition of AuNPs in the system after 240 s. These results demonstrate 10 ppm of PEG 20K and Triton X-100 did not block DOX loading to AuNPs any more than the control (lacking blocking agents), while Tween and BSA inhibited DOX loading to the greatest degree. Additional evidence was obtained by directly comparing the number of DOX molecules sorbed onto AuNP surfaces in the presence or absence of PEG 20K (Fig. S2); critically, the amount of DOX loaded to AuNPs was independent of the presence of PEG 20K (p = 0.0647). Conversely, Tween and BSA may inhibit DOX loading to AuNPs by directly blocking AuNP surfaces, or by decreasing free DOX available to bind AuNPs by binding to DOX themselves.

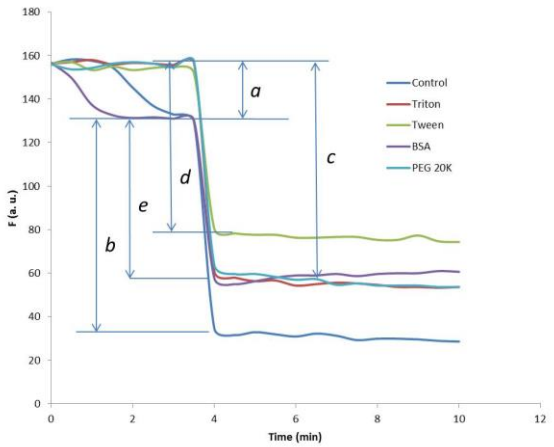


Figure 4. Inhibition of DOX loading to AuNPs by several surface blocking reagents (BSA, Triton X-100, Tween 20, and PEG 20K). AuNPs were introduced after 240s; no surface blocking agents were added to control wells.

Unlike BSA, which is commonly pre-coated to block non-specific adsorption in biochemical assays such as enzyme-linked immunosorbent assays (ELISAs), PEG 20K must be added into the system concurrent with other reagents (DOX and AuNPs). This was evident from our experiment directly testing the effectiveness of PEG 20K and other surface-blocking agents to pre-coat plate wells in a separate step similar to that where BSA is used as a blocking agent of ELISA plates. In our experiment, plate wells were treated with 1% (*v/v*) BSA, PEG 20K, Tween 40, and Triton X-100 (100 ppm) in HEPES buffer (5 mM, pH 7.6) for 30 min at room temperature. After incubation, these pre-coated plate wells were rinsed twice with HEPES buffer prior to DOX fluorescence measurement in HEPES buffer. Two controls were included in this experiment; the first, a positive control, for which the plate wells were treated only with HEPES buffer and the DOX solution contained 10 ppm of PEG 20K. The negative control plate wells contained only HEPES and DOX buffer solutions. DOX sorptive losses remained significant in the PEG-treated plate wells, similar to those not receiving any pre-treatment, presumably as PEG binding to the plate surface was weak, and PEG was unable to adhere to the well surfaces during plate rinsing (Fig. 5). Consequently, the simplest and most effective means of PEG preventing DOX sorptive losses is by including 10 ppm of PEG 20K into the buffer used to prepare DOX solutions. Similar to BSA, Tween and Triton X-100 are also unsuitable blocking agents for pre-treating plate wells. Although BSA did not reduce DOX's sorptive losses when concurrently added (Fig. 3) but rather interfered with DOX loading to AuNPs (Fig. 4), it was however an effective pre-treatment blocker to prevent sorptive losses of DOX, although quite time-consuming and requiring extra steps.

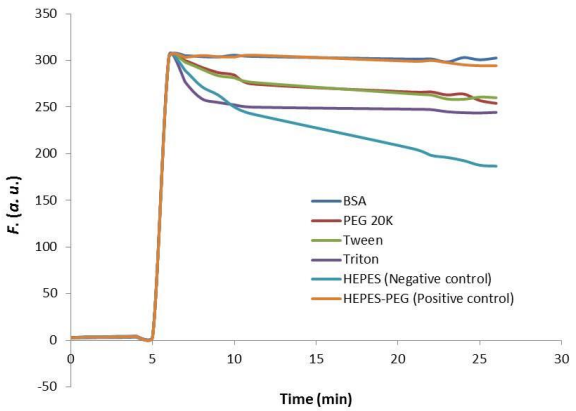


Fig. 5. Relative effectiveness of surface-blocking agents as assessed by monitoring DOX fluorescence in plate wells pre-coated with the various surface-blocking agents. The baseline fluorescence was obtained prior to the addition of DOX solution in HEPES buffer (5 mM) at 5 min.

PEG effect on the photo-degradation of DOX

In addition to sorptive losses, DOX can be photodegraded during storage or analysis upon light exposure.[8-13] Previous studies demonstrated PEG-coated liposomes reduced UVA-induced photodegradation of encapsulated DOX;[29] consequently, we investigated if PEG 20K could protect DOX from blue light (λ = ~ 470 nm) degradation. Photodegradation of DOX was reduced to less than 2% following a 1 min exposure in buffer containing 8000 ppm (or 0.8%) PEG 20K (Fig 6). Even in the presence of only 10 ppm PEG 20K, 74% of intact DOX remained after 1 min light exposure, in contrast to 58% remaining when PEG was absent. Notably, even if 8000 ppm PEG 20K was present, photodegradation continued increasing over time. The protective mechanism of PEG 20K is presumably via decreasing DOX adsorption onto the inner vial surfaces, where the distance between DOX molecules and photons is minimal and photodegradation of DOX is maximized. Interestingly, the protective effect of PEG 20K increased with increased PEG concentrations, a phenomena for which the underlying mechanism is not yet clear but deserving of future investigation. There are several facts contradictory to established mechanisms for PEG enhancing DOX photo-stability; first, PEG 20K does not absorb blue light (Fig. S3).

Second, the DOX concentrations are low (5 μg/mL) and insufficient for concentration-induced self-protection. Third, the PEG 20 K buffer pH was 7.6, while it is acidic buffer that facilitates the ready degradation of DOX.[8,9,12]

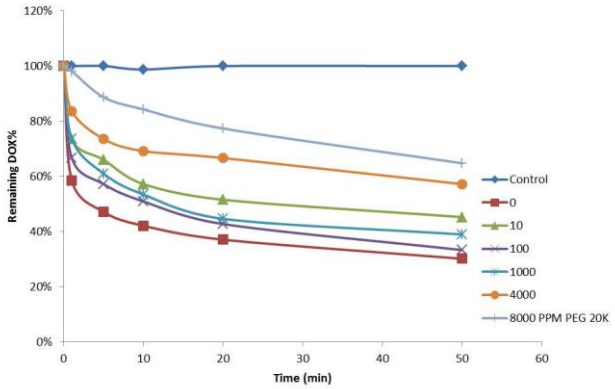


Fig. 6. DOX photodegradation kinetics in solutions containing various concentrations of PEG 20K. The control sample was not exposed to blue light.

Experimental

Chemicals. PEG, Bovine Serum Albumin (BSA), Tween-40, Triton X-100, HEPES and doxorubicin hydrochloride were purchased from Sigma-Aldrich. Ethylene glycol was purchased from Alfa Aesar (Ward Hill, MA) while polystyrene 96-well plates were purchased from Corning Inc. (NY) and microcentrifuge tubes (Cat. No. 02-681-284; Lot No.: 13300434) and ethanol were purchased from Fisher Scientific (Ottawa, ON, Canada). Rh-labeled PEG 10, 000 was purchased from Nanoce, Inc. (Boston, MA). Nanopure 18.2 MΩ-cm water was used in all experimentation.

Doxorubicin adsorption kinetics and isotherm (plates and tubes)

96-well plates. In all experiments, doxorubicin was quantified by fluorescence measurement (excitation/emission: 480 nm/580 nm) using a TECAN Infinite M10000 PRO micro-plate reader. Polystyrene 96-well plates (Costar 3915, Lot No.: 26313022) were used in all quantitation experiments with working volumes of 100 μL in each well, with the exception of isotherm determination where sample volume was increased to 150 μL to offset evaporation during extended (90 min, *n*=3) measurement times.

Doxorubicin stock solution (5 μM in Nanopure water stored in a 1.5-mL amber tube at -20°C) were added to wells containing varying volumes of HEPES buffer (5 mM, pH 7.6 to a final volume of 150 μL in each well) to achieve DOX concentrations ranging from 0.25 to 2.5 μM. DOX adsorbed onto the plate surface was determined by recording the decrease in fluorescence over 90 min. The calibration was performed against a standard curve whose solutions included 10 ppm of PEG 20K.

To determine the role of blocking agents on non-specific DOX adsorption to 96-well plate surfaces, DOX adsorption kinetics were monitored in the presence of various molecular weights (1K, 2K, 4K, 8K, and 20K) and concentrations (ppt to % levels) of PEG. DOX aqueous solutions without any blocking agents added served as the control. Typically, 80 μL of HEPES buffer (5 mM, pH 7.6) were mixed with 10 μL of PEG solution in each well, followed by 10 μL of doxorubicin solution for a final volume in each well of 100 μL. The mixture was gently mixed before recording fluorescent signals over defined intervals (*i.e.*, 6 - 10 min). The identical procedure was repeated for other surface blocking reagents including BSA, Tween-40, and Triton X-100.

The kinetics and capacity of citrate-stabilized AuNPs for DOX adsorption in the presence or absence of blocking reagents (n = 3) was evaluated by fluorescence change over time before and after blocker addition. The final volume of solution in each well was 100 μL, comprising 70 μL of 5 mM HEPES buffer, 10 μL of surface-blocking reagent solution (PEG, BSA, Tween-40, or Triton X-100), 10 μL of DOX stock solution (5 μM in Nanopure water) and 10 μL of (10 nM) AuNP solution.

Microcentrifuge tubes. PEG was evaluated as a blocking agent against doxorubicin adsorption onto surfaces of microcentrifuge tubes (n = 3). Briefly, 10 μL of PEG 20K (1000 ppm) was added to 80 μL of Nanopure water with varying concentrations of NaCl (0, 15, 30, 60, 90, 120, 150 mM) and gently mixed by shaking (control tubes contained an additional 10 μL of HEPES buffer instead of PEG solution). To this mixture, 10 μL of doxorubicin stock solution (75 μM) was added into the tubes and gently mixed again. After 10 min, 10 μL of this mixture was combined with 90 μL of HEPES-PEG buffer (PEG 20K: 10 ppm) in the 96-well plate for fluorescence measurements. In this experiment, the HEPES-PEG buffer was used to prevent DOX adsorption to the plate wells, so our results only reflect adsorption to microcentrifuge tube surfaces.

Doxorubicin Degradation Studies

The potential protective ability of PEG to inhibit the photodegradation of DOX was evaluated using a Safe Imager™ Blue Light Transilluminator. Briefly 1 mL of DOX stock solution was added to a 10 mL clear glass vial (n = 3). Increasing concentrations of PEG 20K were added to achieve a final volume of 1.5 mL before solutions were mixed and capped. Vials containing DOX-PEG were then exposed to the blue light source (λ = 470 nm) from the Transilluminator for increasing time intervals with doxorubicin fluorescence measured kinetically (10 μL of sample solution and 90 μL of HEPES-PEG buffer in the plate well).

Conclusions

In summary, this work demonstrates small amounts of PEG (ppm level) can effectively protect DOX from sorptive losses, and to a limited extent, from photodegradation. Collectively, our data suggests PEG holds promise for use in storage, transportation, and accurate evaluations of drug delivery systems by reducing non-specific adsorption, a common

experimental artifact and analytical challenge in drug analysis. Therefore, this work has wide implications for drug analysis in the pharmaceutical industry and has additional application for environmental scientists.

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Notes and references

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† Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.
Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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