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Original Research Paper

Stability of freeze-dried pH-responsive dextrin nanogels containing doxorubicin

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ABSTRACT

Induction of non-specific toxicities by doxorubicin (DOX) has restricted conventional DOX-based chemotherapy. pH-responsive dextrin nanogels (DNGs) have been fabricated in order to incorporate and deliver DOX to specific (targeted) sites. However, adequate stability studies of DOX-loaded DNGs are required for selection of storage conditions. The aim of this study was therefore to evaluate the accelerated (25 °C/60% RH) and long-term (5 °C) stability of DNGs prepared with formaldehyde (FDNGs) and glyoxal (GDNGs) as cross-linker by determining the change in their physicochemical properties. The mean diameter decreased with time during long-term storage. The drug content between freshly prepared (initial day) and after storage at 5 °C for 180 days of DOX-loaded FDNGs and DOX-loaded GDNGs was not significantly different ($p > 0.05$), but decreased after storage under the accelerated condition. The release of DOX from all DNGs was pH-dependent. However, DNGs kept under the accelerated condition showed higher amount of DOX release than those stored at 5 °C and the freshly prepared ones. The results indicate that the stability of DNGs could be improved by their storage at 5 °C.

1. Introduction

Cancer is a major cause of mortality worldwide with 8.2 million people being affected in 2012 [1]. Major clinical treatments for cancer include surgery, radiation, and chemotherapy, with chemotherapy being the major form. However, chemotherapy is a major form of management of cancer patients enlisting the used drugs to kill cancer cells. Among such drugs, the anthraquinonedoxorubicin (DOX) is a frontline

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chemotherapeutic agent used for treatment of several forms of cancer. Its mechanism of action is to inhibit DNA polymerases and topoisomerases and block the cell cycle, usually resulting in the induction of apoptosis in tumor cells [2,3]. Despite its efficacy, the clinical use of unformulated (free) DOX is limited due to development of progressive cardiomyopathy with apoptosis induction in cardiomyocytes by activation of p53 protein and reactive oxygen species leading to congestive heart failure [4]. In addressing this problem, a variety of innovative approaches to entrap this drug in nanocarriers and hopefully achieve site-specific delivery has been developed.

Among these approaches, pH-responsive nanocarriers have been previously exploited for targeted delivery of drugs. Due to its biocompatibility and degradability [5], dextrin is frequently chosen for nanogels formulating in order to circumvent carrier toxicity. It is a saccharide-based polymer containing D-glucose units linked by α-(1→4) glycosidic bonds, and considerable quantities of hydroxyl groups that are readily modified. Regarding biomedical application, dextrin is employed as a drug delivery system [5–9] and as a scaffold material [10,11]. pH-responsive dextrin nanogels (DNGs) are cross-linked dextrin networks fabricated by incorporating pH-responsive bonds, namely acetal bonds, into their structure. These bonds are used as linkers to immobilize anti-tumor drugs within the carrier matrix. In this system, DNGs are delivered to the tumor site *via* the enhanced permeability and retention (EPR) phenomenon [12,13]. DNGs are stable at physiological pH but could be destabilized and release the drug under mild acidic conditions at the target neoplastic site, resulting in enhanced therapeutic efficacy and reduced side-effects to normal tissue. Despite DNGs providing many benefits, the challenge remains in producing highly stable forms of encapsulated DOX and maintaining the long-term pH-responsive behavior. Knowledge of the stability helps in selecting appropriate formulation and packaging as well as providing suitable storage conditions and shelf-life, which is essential for regulatory documentation [14].

The purpose of this research is to investigate the long-term stability and accelerated stability of two different types of pH-responsive DNGs, that is, FDNGs and GDNGs which were formulated using formaldehyde and glyoxal as a cross-linker, respectively. In addition, the effects of various types and quantities of cross-linker on stability were also studied. The changes of properties namely mean diameter, ζ-potential, chemical structure, drug remaining, pH-responsive behavior and amount of drug release in both DNGs after storage over a period of 6 months were evaluated in order to elucidate the optimal conditions for DNG stability during storage for future application.

2. Materials and methods

2.1. Materials

Dextrin (molecular weight of 1400 Da) was a gift from Siam Modified Starch Co., Ltd. (Pathumthani, Thailand). Glyoxal, ethanol and doxorubicin hydrochloride (DOX) were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). Hydrochloric acid, formaldehyde and n-hexane were purchased from RCI Labscan (Bangkok, Thailand). Tween® 80 and Span® 80 were purchased from P.C. Drug Center Co., Ltd. (Bangkok, Thailand). Deionized water was used throughout the study.

2.2. Preparation of dextrin nanogels

DOX loaded-DNGs were prepared as described previously by our group [15] with some modifications. Water-in-hexane emulsions were prepared, in order to form a nanoemulsion template, using 7% (w/w) mixture of Span® 80/Tween® 80 as emulsifier. Dextrin and DOX were dissolved in the water phase to obtain the final concentration of 5% (w/w) and 0.2 mg/mL, respectively. The water phase was added to the emulsion template and ultrasonicated for 1 minute to form nanoemulsions. After the nanoemulsions were obtained, different concentrations of cross-linking agent (that is, formaldehyde or glyoxal) were added immediately to achieve mole ratios of dextrin to cross-linking agent of 4:1, 10:1, 15:1 and 20:1. The mixtures were homogenized via ultrasonication (UP400S, Hielscher, Germany) with 100% amplitude of ultrasound power (400 W, 24 KHz) for 30 min. The obtained nanoemulsions were then stirred with a magnetic stirrer for 12 h to continue the cross-linking reaction. DNGs were precipitated from the nanoemulsions by adding 99% (v/v) ethanol and washed 3 times with ethanol and finally rinsed with deionized water. Subsequently, the DNGs were freeze-dried for 24 h. The dried DNGs obtained from the freeze-drying process were packaged in zip-lock bags and kept at 4 °C until further analysis.

2.3. Stability study

The dried DNGs were kept under two conditions – 25 °C ± 2 °C/60% ± 5%RH (accelerated conditions; in stability chamber) and 5 ± 3 °C (long term condition; in refrigerator), for 6 months before further investigation.

2.4. Particle size and ζ-potential determination

Before measurement, DNGs were dispersed with phosphate buffer (pH 7.4, 6.8 and 5) and filtered through a 0.45-μm membrane. Zetasizer Nano-ZS (Malvern Instruments, UK) equipped with a He—Ne laser beam operating at a wavelength of 633 nm and a detector fixed at a scattering angle of 173°, was used to determine the hydrodynamic diameter and ζ-potential of DNGs at 25 °C. Measurements were performed three times.

2.5. Morphological observation of DNGs

Morphological analysis of DNGs was carried out on a transmission electron microscope (TEM; model JEM-1230, JOEL Corp., Japan). TEM analyses were performed by sample mounting on a copper glider grid of 3.5 mm with a single aperture, adsorbed with filter paper and dried at ambient temperature, prior to TEM examination.

2.6. ^{13}C nuclear magnetic resonance spectroscopy (^{13}C NMR)

The samples were dissolved in deuterium oxide. The ^{13}C NMR spectra of samples were recorded on NMR spectroscopy (model

ADVANCE 300, Bruker, Germany) with deuterium oxide as the solvent. The chemical shifts were given in δ (ppm).

2.7. Drug content determination

The DNG dispersion was mixed with 1.0 N HCl and stirred for 12 h. Subsequently, the suspensions were filtered through 0.45-μm cellulose acetate membrane. The DOX concentration in DNGs was measured using UV absorbance at 495 nm with a UV/vis spectrophotometer (model T60U, PG Instrument Ltd., England). All measurements were performed in triplicate. DOX concentration was then calculated based on a standard curve of known amounts of DOX in 0.1 N HCl. Drug content and drug remaining were defined as:

$$\text{Drug content}(\%) = \frac{\text{Weight of loaded drug}(mg) \times 100}{\text{Weight of drug} - \text{loaded nanogels}(mg)} \quad (1)$$

$$\text{Drug remaining}(\%) = \frac{\text{Drug content after stability test}(\%) \times 100}{\text{Drug content at initial day}(\%)} \quad (2)$$

2.8. In vitro drug release study

The *in vitro* release of DOX-loaded DNGs were investigated using the dialysis method [15]. Briefly, DOX-loaded DNGs were added to a dialysis membrane bag (Cellu-Sep T2 MWCO 6–8 kDa; Membrane Filtration Products Inc., Braine-l'Alleud, Belgium), and then immersed in phosphate buffer (25 mL) and shaken horizontally (100 rpm) at 37 °C using an environmental shaker incubator (model ES-20, Orbital Shaker-Incubator, Biosan, Latvia). At certain time points, the outer phase of the dialysis membrane bag was harvested and replaced with fresh buffer. The concentration of DOX in the collected samples was analyzed under UV/vis absorbance mode at 495 nm.

2.9. Statistical analysis

Data were analyzed using SPSS version 11.5 for Windows (SPSS Inc., USA). The results were represented as mean ± standard deviation (SD). Analysis of variance (One-way ANOVA) with Scheffé or Games–Howell *post hoc* test was performed to evaluate difference among the groups. The statistical significance was set at $p < 0.05$.

3. Results and discussion

As previously reported [16], pH-responsive DNGs were prepared by an emulsion cross-linking technique using glyoxal or formaldehyde as a cross-linker. In this method, nanogels were formed in the nanoemulsion droplet by simultaneously cross-linking and creating 3-dimensional structures. However, it is known that nanogels are prone to aggregation, leading to an increase in particle size and loss of certain properties with time [17]. Therefore, the physicochemical properties, amount of encapsulated drug and drug release behavior over the process of storage were used as indicators to evaluate the accelerated and long-term stability of FDNGs and GDNGs, namely 25 °C/60% RH and 5 °C, respectively.

3.1. Changes in physicochemical properties

The ability of DOX-loaded FDNGs and GDNGs to maintain their sizes over a long storage period was evaluated. Fig. 1 shows that size and zeta-potential of DOX-loaded FDNGs and GDNGs changed after storage at 25 °C/60% RH and 5 °C for 180 days. The results indicate that the trends of mean diameter and ζ-potential change were varied; they tended to decrease with time, reflecting the different changes during different stages in long-term storage. Minor change in the size of DNGs kept at 5 °C was observed, whereas the size of DNGs keeping at 25 °C/60% RH showed an apparent change over the time period investigated. The change of size was probably due to the unstable linkage that could be broken during storage as a result of exposure to moisture; however, storing at 5 °C seemed to increase conservation of size of nanogels.

3.2. Changes in chemical structure

Figs. 2 and 3 shows the ^{13}C NMR spectra of freshly prepared FDNGs and GDNGs and those after storage at 25 °C/60% RH and

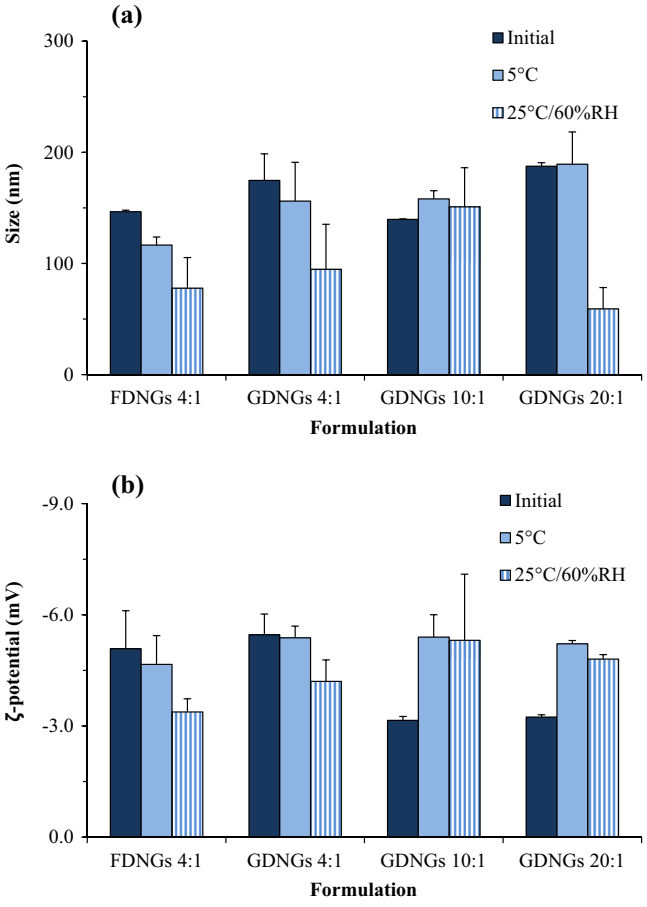

Fig. 1 – (a) Size and (b) ζ-potential of DOX-loaded FDNGs (4:1), GDNGs (4:1), (10:1) and (20:1) at initial day and after storage at 25 °C/60% RH and 5 °C for 180 days.

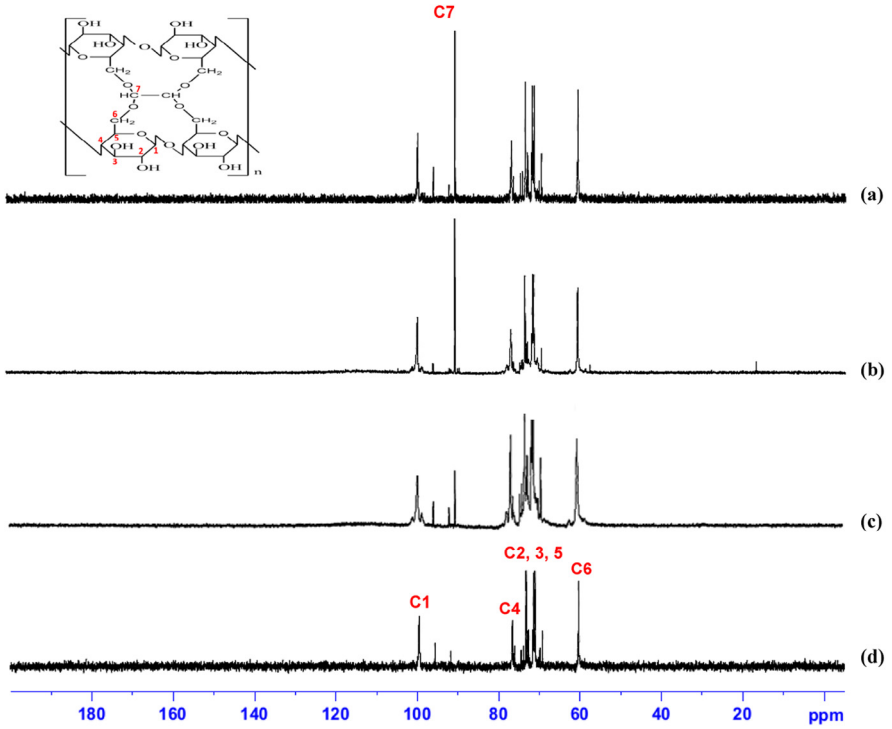

Fig. 2 – ^{13}C NMR spectra of GDNGs at a molar ratio of dextrin to glyoxal of 4:1 at (a) initial day, (b) after storage at 5 °C, and (c) 25 °C/60% RH, compared to (d) native dextrin.

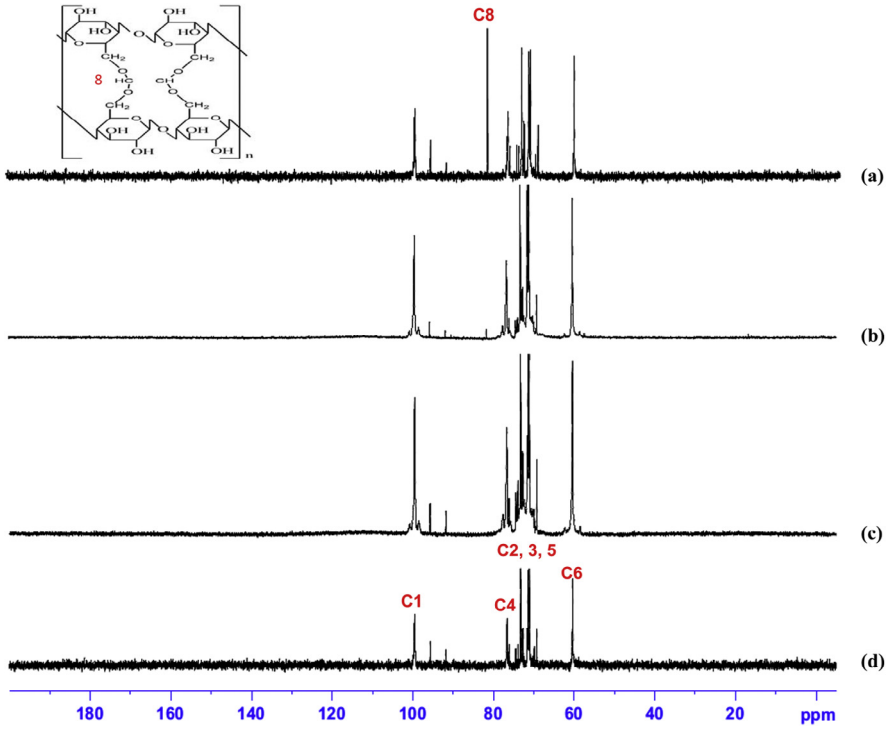

Fig. 3 – ^{13}C NMR spectra of FDNGs at a molar ratio of dextrin to formaldehyde of 4:1 at (a) initial day, (b) after storage at 5 °C, and (c) 25 °C/60% RH, compared to (d) native dextrin.

5 °C, fabricated at a mole ratio of dextrin to formaldehyde or glyoxal of 4:1. The chemical shifts at 99.52 ppm (C1), 76.62 ppm (C4), 73.27–71.10 ppm (C2, C3, C5), and 60.39 ppm (C6) verified a presence of dextrin molecule as reported previously [18–20]. The signals at 90 ppm (C7) of GDNGs (Fig. 2a) and 84 ppm (C8) of FDNGs (Fig. 3a) were assigned to the formation of acetal linkage [21]. After storage at 25 °C/60% RH and 5 °C, the characteristic peaks of this acetal linkage of GDNGs (Fig. 2c) were decreased when compared to freshly prepared ones, but no signal at 84 ppm of FDNGs (Fig. 3c) was observed. The absence of these peaks is probably due to destabilization and degradation of acetal bonds after storage under the accelerated conditions, perhaps by the moisture in the humid condition. However, the NMR signals of GDNGs that were kept at 5 °C were similar to freshly prepared ones, indicating that storage at 5 °C could retain nanogel stability long-term. On the other hand, the small signal was observed at 84 ppm for FDNGs, indicating that the acetal bonds, which were formed by formaldehyde, were less stable than that formed by glyoxal.

3.3. *Changes in encapsulated drug*

The ability to retain encapsulated drug during storage is also critical to the development of a drug carrier. The drug remaining in nanogels (both FDNGs and GDNGs) was evaluated to determine the long-term stability of these formulations. The stability of DOX-loaded FDNGs and GDNGs is shown in Fig. 4. There was no significant difference observed in the encapsulation efficiency between freshly-prepared (initial day) DOX-loaded FDNGs and DOX-loaded GDNGs and those after storage at 5 °C for 180 days ($p > 0.05$). However, the encapsulation efficiency decreased after storage at accelerated condition, 25 °C/60% RH (data not shown). According to the literature, DOX is stable in the solid state at 2–8 °C (protected from light), and the degradation rate of DOX increases with increasing temperature [22–24]. Moreover, the amount of glyoxal also affected DOX stability; DOX was less stable when the dextrin to glyoxal ratio was increased from 4:1 to 20:1. It is possible that DOX cannot be completely entrapped in nanogels with lower quantities of cross-linker; therefore, DOX located at the surface of the nanogels is easily degraded by the environment.

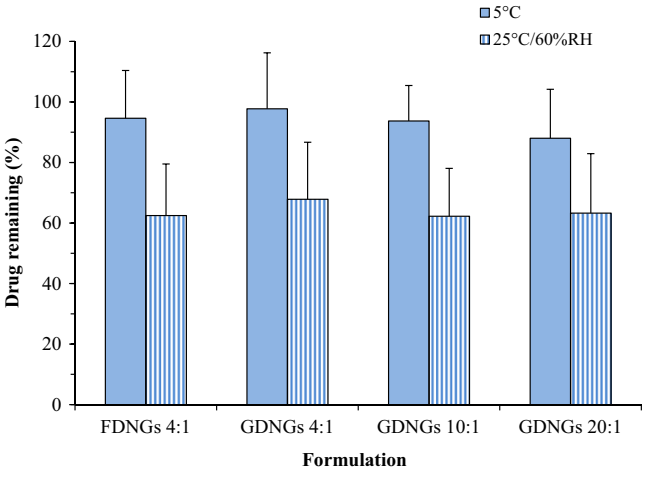

Fig. 4 – Drug remaining in DOX-loaded FDNGs (4:1), GDNGs (4:1), (10:1) and (20:1) at initial day and after storage at 25 °C/60% RH and 5 °C for 180 days.

3.4. *Changes in pH-responsive behavior and DOX release*

The mechanism controlling the release of DOX incorporated in DNGs is mainly attributed to pH-induced structural changes. This behavior has been ascribed to the hydrolysis of acetal bonds in the DNG structure under mildly acidic conditions, resulting in destabilization of the structural integrity of DNGs that could accelerate DOX release at pH below 7 [21]. In order to verify the hypothesis that DOX was released due to the destabilization of DNG structure, the morphology of FDNGs and GDNGs at a mole ratio of dextrin to cross-linker of 4:1 at each pH, (5, 6.8 and 7.4) was examined by TEM. The TEM images showed deformation and fracture in both FDNG and GDNG structures; the size of DNGs decreased with decreasing pH (Fig. 5). These results confirmed that the change of nanogel structure by acid hydrolysis was attributed to the difference in drug release under different pHs.

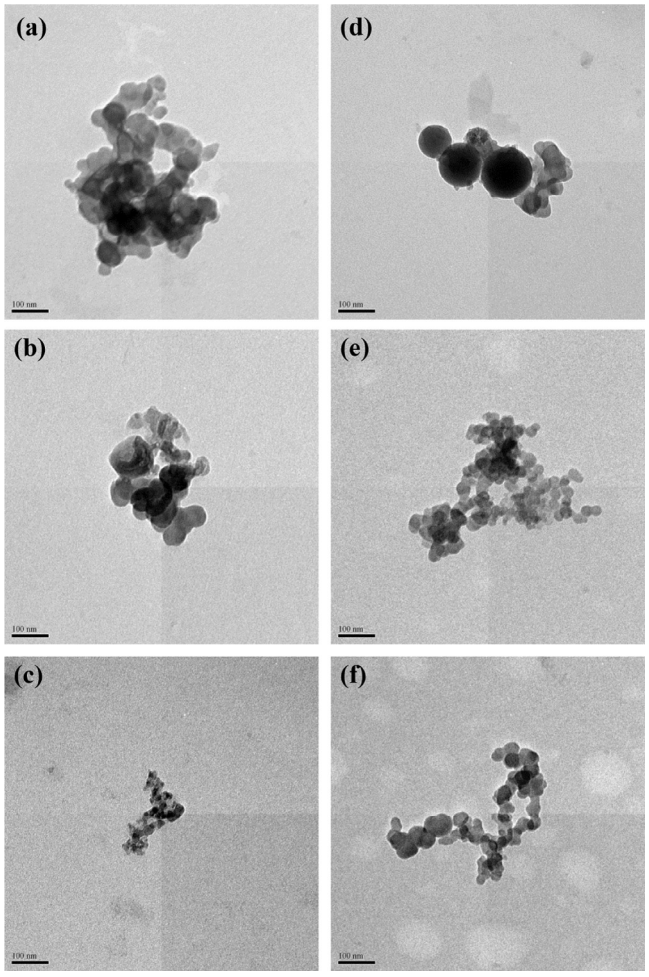

Fig. 5 – TEM micrographs of DOX-loaded FDNGs at a mole ratio of dextrin to formaldehyde of 4:1 in (a) pH 7.4, (b) pH 6.8, (c) pH 5 phosphate buffer, and DOX-loaded GDNGs at a mole ratio of dextrin to glyoxal of 4:1 in (d) pH 7.4, (e) pH 6.8, (f) pH 5 phosphate buffer.

The change in pH dependency of both FDNGs and GDNGs after storage was investigated. Fig. 6 shows the amount of DOX released at different pHs, within 24 h, from FDNGs at a mole ratio of dextrin to formaldehyde of 4:1 and GDNGs at a mole ratio of dextrin to glyoxal of 4:1 and 20:1, before and after 6-month storage. All DNGs demonstrated pH-dependent drug release properties. Drug release was slow at physiological pH but increased significantly in acidic medium. In addition, type and amount of cross-linker also affected the release of drug, similar to freshly prepared ones (Fig. 6). However, DNGs tested under accelerated condition (25 °C/60% RH) showed higher amount of DOX release than those kept at 5 °C and the freshly-prepared ones. At pH 5, about 98%, 60% and 50% of DOX were released from GDNGs at mole ratio of dextrin to glyoxal of 20:1 kept at 25 °C/60% RH, 5 °C and freshly-prepared GDNGs, respectively. The results indicated that the acetal bond in DNG structure was unstable, easily hydrolyzed in solution when stored under the accelerated condition for 6 months. These changes related well to the corresponding NMR spectra.

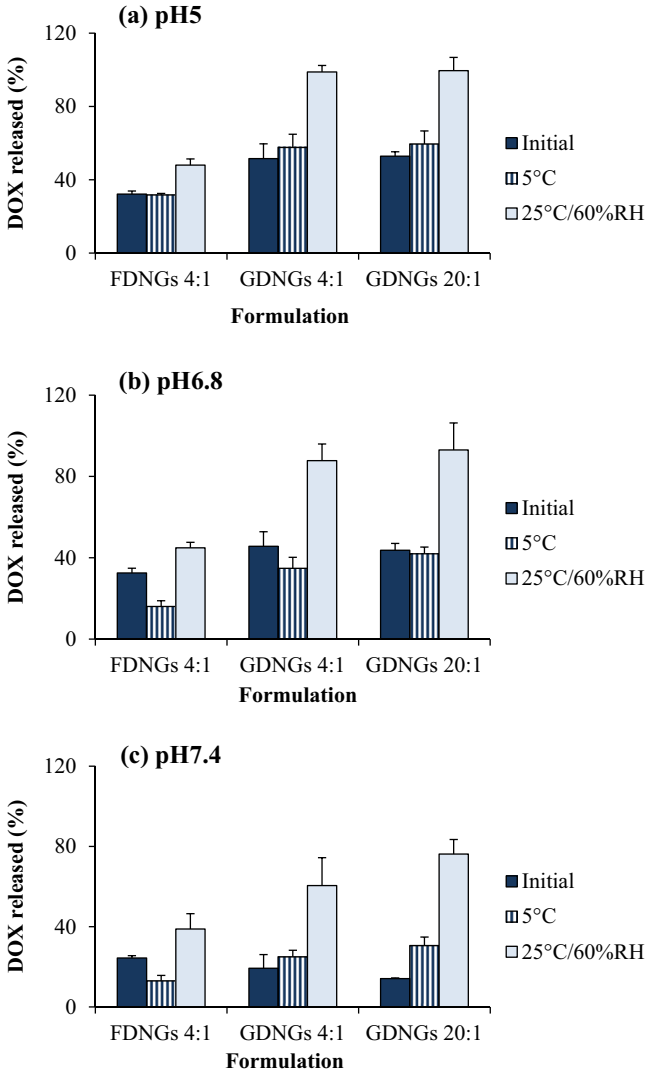

Fig. 6 – pH-dependent release of DOX from FDNGs (4:1), GDNGs (4:1) and (20:1) at 37 °C in (a) pH 5, (b) pH 6.8, (c) pH 7.4 phosphate buffer, after storage under the long-term (5 °C) and accelerated (25 °C/60% RH) conditions.

4. Conclusion

The stability of both FDNGs and GDNG was examined by measuring the change of their physical properties over a period of 6 months under different conditions, 5 °C and 25 °C/60% RH. Under accelerated condition (25 °C/60% RH), both FDNGs and GDNGs were found to be unstable; the particle size and amount of encapsulated DOX decreased over time. In contrast, the amount of DOX release increased at all pH conditions, compared with those kept at 5 °C and the freshly prepared ones. The results of NMR demonstrated that the destabilization and degradation of acetal bonds occurred after storage under the accelerated condition. In addition, the long-term stability of DNGs is affected by type of cross-linker, with GDNGs being more stable than FDNGs. The stability of DNGs can be manipulated by storage at 5 °C.

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