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PII: S0141-8130(19)40483-2

DOI: <https://doi.org/10.1016/j.ijbiomac.2020.02.042>

Reference: BIOMAC 14659

To appear in: *International Journal of Biological Macromolecules*

Received date: 19 December 2019

Revised date: 30 January 2020

Accepted date: 5 February 2020

Please cite this article as: S.R. Batool, M.A. Nazeer, D. Ekinici, et al., Multifunctional alginate-based hydrogel with reversible crosslinking for controlled therapeutics delivery, *International Journal of Biological Macromolecules*(2020), <https://doi.org/10.1016/j.ijbiomac.2020.02.042>

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Multifunctional Alginate-based Hydrogel with Reversible Crosslinking for Controlled Therapeutics Delivery

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Abstract

Glycan-based alginate hydrogels have great potential in creating new vehicles with responsive behavior and tunable properties for biomedicine. However, precise control and tunability in properties present major barrier for clinical translation of these materials. Here, we report the synthesis of pH responsive anthracene modified glycan-based hydrogels for selective release of therapeutic molecules. Hydrogels were crosslinked through simultaneous photopolymerization of vinyl groups and photodimerization of anthracene. Incorporation of anthracene into these gels lead to reversible control on crosslinking and transition between gel/sol states through dimerization/dedimerization of anthracene groups. Chemotherapeutic drug doxorubicin-loaded hydrogels were then tested in a cancer mimetic microenvironment where 85% of the drug was released from anthracene-conjugated hydrogels at pH 2 for 6 days. Control on gelation with anthracene incorporation was observed through alterations in modulus, where storage modulus was increased two- and five-fold with anthracene conjugation during photopolymerization and photodimerization, respectively. Furthermore, cell survival analysis revealed that anthracene conjugation could selectively compromise cancer cell viability without inducing significant toxicity on healthy fibroblasts. This study combines light-induced control of crosslink density due to anthracene and pH-triggered therapeutics delivery with alginate. The approach would be applicable for systems where multiple control is required with high precision.

Key words: anthracene-functional hydrogel; photodimerization; pH-responsive hydrogel

1. Introduction

Glycans are oligosaccharides or polysaccharides found in bacteria, archaea, and eukaryotes, consisting of O-glycosidic linkage of monosaccharides. For instance, cellulose is a glycan consisting of β -1,4- glycosidic linked D-glucose units and similarly, alginate residues α -L-guluronic (G-block) and β -D-mannuronic acid (M-block) linked through 1-4 glycosidic linkages [1]. Alginate is a natural anionic and linear glycan made up of block copolymer of GG, MM, and MG blocks. It can be crosslinked ionically with divalent cations (Ca^{+2} or Mg^{+2}) or covalently through chemical alteration of carboxyl groups ($-\text{COOH}$) via carbodiimide chemistry resulting in a stable hydrogel network [2, 3]. Alginate is one of the most commonly being used glycan for various biomedical applications due to its biocompatibility, non-immunogenicity, non-toxicity, and biodegradable nature [4]. Hence, alginate-based hydrogel has numerous applications in cell transplantation, angiogenesis, cell encapsulation, stem cell culture, and controlled drug delivery systems [5, 6].

Physically and/or chemically crosslinked 3D polymeric hydrogels have similarity with the microenvironment of natural extracellular matrix. Due to the presence of hydrophilic groups such as hydroxyl and amine, this 3D network can retain large amount of water [7]. Hydrogels have been classified on the basis of monomer, overall-charge, degradability, and their responsiveness to altered environmental stimuli [8]. Among all, stimuli-responsive hydrogels have been considered for sustained drug delivery due to their diverse properties including porosity, mechanical stability, overall charge, electrostatic interactions with drug molecules and more specifically the ability to change their function depending on one or more stimuli [9]. In particular, pH responsive hydrogels are suitable for targeted and sustained drug delivery due to differences in pH among various tissues and organs, for instance, pH of endosomal cancer cells is around 5.0-6.0 and healthy cells have a typical pH of 7.3-7.5 [10].

Characteristically, pH responsive hydrogels demonstrate reversible swelling/deswelling behavior with a change in pH. This response occurs due to acidic or basic moieties present in hydrogel network which play a role in accepting or releasing protons with alterations in environmental pH [11].

Doxorubicin (DOX) is a well-known antitumor agent, extensively being used for cancer therapy to treat different types of cancers including breast, stomach, lung, and bladder [12]. The major drawback associated with low dose DOX is its limited internalization by cancer cells. Employing its high concentration can compromise cancer cell survival but at the same time generates severe side effects for healthy cells and tissues such as cardiotoxicity and hypersensitivity [13]. Therefore, it is critical to develop a system that has selective influence on cancer cells to promote apoptosis. For this, an ideal candidate can be a pH responsive system that can promote temporal release of therapeutic molecules being modulated through pH.

Anthracene, a photo-reactive molecule, has the ability to form chemical crosslinks through dimerization in the absence of a photoinitiator with the aid of heat treatment or exposure of light at a specific wavelength. It can dimerize (at ~365 nm) and dedimerize (at ~254 nm) and this property can be exploited to synthesize hydrogels with reversible control on crosslinking [14]. Anthracene and its derivatives such as; anthracycline, mitoxantrone and bisantrene have critical biological activities against cancer [15]. Previously, it was reported that, an anthracene derivative, MHY-412, had a major role to promote apoptosis of breast cancer cells and anthracene was found to be non-toxic to healthy cells as well [16, 17] and anthracene conjugated PEG-alginate and PEG-hyaluronan photogel showed potential to be used as a hydrogel scaffold [14].

Although, in some previous studies, anthracene was conjugated with polymers and crosslinking was performed through dimerization of anthracene under UV, none of these

studies considered crosslinking of polymers with simultaneous dimerization and covalent crosslinking with UV light or synthesized anthracene-conjugated glycan-based alginate hydrogel. To the best of our knowledge, synthesis and application of anthracene-conjugated methacrylated-alginate (anth-MA-alginate) hydrogel through photopolymerization (at $\lambda=514$ nm) and/or photodimerization (at $\lambda=365$ nm) has not yet been reported. Furthermore, we also believe that we can exploit anticancer property of anthracene in hydrogel structure and it can have biological effects on cancer cells.

In this study, we synthesized methacrylated alginate (MA-alginate) through esterification reaction and conjugated it with 9-Aminoanthracene (9-AA) by using EDC/NHS chemistry. Dual crosslinking was performed simultaneously through free radical photopolymerization of vinyl groups and photodimerization of anthracene as illustrated in **Fig. 1**. We observed improvement in mechanical properties of photopolymerized hydrogel after anthracene incorporation. Furthermore, the release of DOX from anth-MA-alginate hydrogel was sustained and improved at acidic pH, mimicking the cancer tissue microenvironment. Finally, our cell viability analysis revealed that cancer cell survival in anth-MA-alginate hydrogel was lower than that was observed in MA-alginate. Remarkably, healthy cells (NIH-3T3) viability in MA-alginate and anthracene-based hydrogel was $\sim 100\%$. This approach has potential to enable the development of glycan-based vehicles with embedded functions for therapeutic cargo delivery towards cancer cell targeted microenvironment without compromising the survival of healthy cells.

2. Experimental

2.1. Materials

Sodium alginate (38 kDa, ≈ 250 cP, M/G ratio: 0.85), anthracene, methacrylic anhydride, Eosin-Y, N-vinyl-2-pyrrolidinone (NVP), acetone, phosphate buffer saline (PBS) tablets, 2-(N-morpholino)ethanesulfonic acid (MES), fluorescein diacetate (FDA) and propidium iodide

(PI) were purchased from Sigma-Aldrich (St. Louis, MO). Glacial acetic acid (99.7%) and anhydrous tin chloride were received from Alfa Aesar (Ward Hill, MA). NaOH, HCl (37%), conc. HNO₃, ethanol, and triethanolamine (TEA) were obtained from Merck. Dulbecco's modified Eagle medium (DMEM, 41966), fetal bovine serum (FBS), penicillin/streptomycin, L-glutamine (200 mM), and trypsin-3 EDTA (25 %) were procured from Gibco (Grand Island, NY). CellTiter-Glo® luminescent cell viability assay was purchased from Promega (Madison, WI, USA). 2, 4, 6-Trinitrobenzenesulfonic acid (TNBSA), N-hydroxysuccinimide (NHS) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were received from Thermo Fisher Scientific (Waltham, Mass) while DOX was purchased from Carbosynth (Compton, UK). All the chemicals were used as received without further purification. Triple distilled water (Milli-Q system water-18.2 MΩ) was used for all experiments.

2.2. Synthesis of 9-Aminoanthracene

9-AA was synthesized using two-step procedure as describe previously [18, 19]. In the first step, 9-Nitroanthracene (9-NA) was synthesized by preparing anthracene solution (44.8 mmol) in glacial acetic acid (32 mL) followed by dropwise addition of conc. nitric acid (3.2 mL) in a 2 neck round bottom flask equipped with a thermometer and addition funnel. A clear solution was obtained after vigorous stirring for 1 h at 25±2°C. Subsequently, a mixture of glacial acetic acid and conc. HCl solution (40 mL, 1/1 by volume) was added slowly under continuous stirring to obtain yellow precipitates. Obtained crude product was filtered, washed first with glacial acetic acid and then DI water until the washing filtrate became neutral. The resulting yellow precipitate was triturated with warm (60±2°C) NaOH solution (10% w/w). The product was filtered, washed with warm NaOH solution (10%) and ultimately with DI water and air dried. The obtained 9-NA was further purified by recrystallization process in glacial acetic acid. For this, each gram of 9-NA was dissolved in 10 mL of preheated glacial

acetic acid and then cooled down to room temperature (RT). In last, crystals were filtered and washed once with glacial acetic acid and twice with hexane, and finally air dried to obtain pure 9-NA. In the second step, 9-AA was synthesized from 9-NA in the presence of a reducing agent. For this, 9-NA (15.68 mmol) was introduced in a reaction flask with 70 mL of glacial acetic acid under continuous stirring at $80\pm 2^{\circ}\text{C}$ for 90 min. Subsequently, a slurry mixture of SnCl_2 (73.83 mmol) and conc. HCl (54 mL) was added slowly in the reaction vessel and stirring was continued for additional 30 min at $80\pm 2^{\circ}\text{C}$. Yellow precipitates were observed at the end of the reaction. Reaction mixture was cooled down to RT, filtered and washed with conc. HCl (15 mL). Finally, the mixture was treated with NaOH solution (5% w/w) for 15 min under mild stirring. Solid residue was collected through filtration and rinsed thoroughly with DI water until neutral filtrate was obtained. The resulting product was dried in air oven at $60\pm 2^{\circ}\text{C}$ and analyzed through ^1H nuclear magnetic resonance (Nuclear magnetic resonance spectrometer, Agilent VNMRs 500 MHz), ATR-FTIR (Attenuated total reflection-Fourier transform infrared spectroscopy, Thermo-Scientific iS50 equipped with ATR), UV-Vis spectroscopy (UV-Vis-NIR Shimadzu UV-3600), and TNBS assay.

2.3.MA-alginate and anth-MA-alginate synthesis

MA-alginate was synthesized through esterification reaction as described previously with minor modifications [20]. Briefly, sodium alginate solution (2.5% w/v) was prepared in 30 mL of DI water through overnight stirring. Methacrylic anhydride (10 mL) was added slowly into the solution of sodium alginate and pH was adjusted at ~ 7.0 periodically by using NaOH (5N) solution. Reaction was continued for 72 h, and MA-alginate was obtained through coagulation in ethanol followed by filtration and washing with ethanol twice. Dried MA-alginate was obtained through lyophilization. 9-AA was used to modify the synthesized MA-alginate through carbodiimide chemistry. For this, MA-alginate (2.5% w/v) solution was prepared in 10 mL of MES buffer containing MES (0.1 M), NaCl (0.5 M) and NaOH to

adjust the pH at 5.5 [14]. EDC (0.5 mmol) and NHS (1 mmol) were added to MA-alginate solution and stirred vigorously for 1 h to activate the carboxyl groups of alginate. The solution of 9-AA (0.05 mmol) in acetone (5 mL) was added dropwise to conjugate 9-AA with the activated carboxylic groups of MA-alginate. After 24 h of stirring at room temperature, the resulting mixture was dialyzed against DI water by using dialysis tube (14 kDa cutoff) for 3 days while changing water twice a day to remove unreacted reagents and impurities. Anth-MA-alginate was obtained through lyophilization. Further purification of anth-MA-alginate was carried out by coagulating its solution in ethanol. Precipitates were filtered, dried at $37\pm 2^{\circ}\text{C}$ in an incubator to remove residual ethanol, re-dissolved in water and dried through lyophilization.

2.4. Photocrosslinked hydrogel formation

Hydrogel was made via free radical photopolymerization under argon laser (argon ion laser, 514 nm, Coherent, Santa Clara, CA) using MA-alginate and anth-MA-alginate prepolymer solutions (**Fig. 2-step-2A, 2B**). Prepolymer solution (2% w/v) was prepared in 2 mL of PBS containing 20 μL of Eosin-Y solution (0.5% w/w in NVP) and 100 μL of TEA (5 M). Solution was transferred to custom made plastic mold (0.2 cm^2) and exposed to laser light for 5 min with a power of 100 mW/cm^2 [21, 22]. When light is irradiated on photoinitiator (Eosin-Y), free radicals are generated and due to the excitation from ground state to triplet state, it protonates TEA (coinitiator). Subsequently, generated α -amino TEA radicals initiate free radical polymerization and react with unsaturated vinyl groups to form a series of crosslinks. NVP was used as an accelerator in this reaction and mechanism is well explained in our previous studies [21, 23]. Upon exposure, pinkish and viscous prepolymer solution transforms to colorless, disk shaped hydrogel. Crosslinked hydrogels were washed with DI water for 3 days to remove unreactive moieties and lyophilized for further studies and characterization. The impact of dual crosslinking (photopolymerization and

photodimerization) on storage moduli was analyzed by making MA-alginate and anth-MA-alginate hydrogels under UV light ($\lambda=365$ nm) equipped with a Rheometer (TA Instruments AR/DHR series, New Castle, DE) (**Fig. 2-step-2C**) in the presence of photoinitiator, coinitiator and accelerator [24]. 315 μL of each prepolymer solution was loaded onto 20 mm quartz plate, working gap was set to be 1000 μm and exposed to UV light with a power of 100 mW/cm^2 . The measurements were carried out in time sweep mode (5 Hz frequency, 1% strain) and storage and loss moduli profiles were recorded as a function of time continuously up to 40 min at ambient temperature. The time required for gelation was estimated through monitoring the elastic and viscous moduli (G' and G''). For dimerization studies only, prepolymer solutions of anth-MA-alginate with varying conc. of 1%, 1.5% and 2% w/v in PBS in the absence of photoinitiator and coinitiator were prepared and run.

2.5. Swelling and deswelling behavior of hydrogel

To assess the pH sensitivity and mechanical stability, reversible swelling/deswelling behavior of hydrogels was evaluated. Dry hydrogels were pre-weighted, equilibrated in DI water and then incubated in pH 2.0 solution to mimic the stomach environment (artificial gastric fluid). The weight of wet hydrogel was recorded every 30 min for 2 h. Subsequently, hydrogels were re-incubated in pH 7.4 solution to mimic the intestine environment (artificial intestinal fluid) and alteration in weight was recorded after every 30 min for 2 h, and this cycle was repeated three times. The swelling ratio Q (**Eq. 1**), corresponding to the change in weight of hydrogels was calculated as described in our previous study [25]:

$$Q = \frac{W_s}{W_d} \quad (1)$$

where, W_s is the weight of swollen or wet hydrogel and W_d is the initial weight of dried hydrogel.

2.6. Drug loading and release studies

Dox was used as a model drug for drug loading and release studies. To load drug, individual hydrogel was incubated in 1 mL of drug solution (1 mg/mL) for 24 h at room temperature under constant shaking (75 rpm). Drug loaded hydrogels were washed with DI water 3 times in order to remove free drug molecules present on the surface. The absorbance of the drug solution was measured by nanodrop (Thermoscientific Nanodrop ND100) at 480 nm. DOX solutions with varying concentration were prepared to generate standard calibration curve. Loading efficiency was calculated by using **Eq. 2**.

$$\text{Loading Efficiency (\%)} = (M_o - M_f) / M_o \times 100 \quad (2)$$

where, M_o is the initial mass of DOX in stock solution and M_f represents the final mass of DOX remaining in the solution after 24 h.

For drug release studies, three different pH values of 7.4, 5.0 and 2.2 were used in PBS medium. The pH was adjusted by using HCl solution (1N) and DOX loaded hydrogels were incubated at $37 \pm 2^\circ\text{C}$ under constant shaking (100 rpm). Drug release at different time intervals was calculated by using **Eq. 3**:

$$M_t = C_t \times V + \sum C_{t-1} \times V_s \quad (3)$$

where, M_t is mass released at time t , C_t is DOX concentration at time t , V is the total volume of solution (10 mL), and V_s is the sample volume (300 μL). M_t value obtained from Eq. 3 can be used to quantify the percent cumulative release of DOX (**Eq. 4**):

$$\text{Percent Mass release} = (M_t - M_\infty) \times 100 \quad (4)$$

where, M_∞ is the total weight of drug loaded into the hydrogel. Sample (300 μL) from each solution was taken for analysis at different time intervals: 1, 2, 3, 4, 6, 24, 48, 72, 96, 120 and 144 h and the final volume of the solution was maintained through addition of the same volume of PBS. The absorbance of the drug was measured at 480 nm by using Nanodrop. Drug concentration was calculated by using standard calibration curve determined through known concentrations of drug solutions.

2.7. *In vitro* cell viability and live/dead assay

The survival of NIH-3T3 mouse fibroblast and human cervical cancer cells in the presence of MA-alginate and anth-MA-alginate hydrogels was assessed through cell viability assay. HeLa cells were kindly provided by the school of medicine, Koc University while NIH-3T3 were obtained from ATCC[®]. Mouse fibroblast and human cervical cancer cells were cultured separately in high-glucose DMEM medium supplemented with 10% FBS, 1% L-glutamine, 1% sodium pyruvate, 1% sodium bicarbonate and 1% penicillin/streptomycin in incubator with 5% CO₂ supply at 37 °C. Prior to cell seeding, MA-alginate and anth-MA-alginate hydrogels were washed with DI water for 3 days at 37±2°C and sterilized from both sides through UV exposure for 30 min per each side. Afterwards, HeLa (cell number=20,000) and NIH-3T3 cells (cell number=10,000) were seeded onto hydrogels in 24 well plates by using 800 µL of DMEM medium. Control group was also prepared by seeding the same number of respective cells in well plate in the absence of hydrogels. Cell viability was measured on day-1 and day-4 through adenosine triphosphate (ATP) based CellTiter-Glo (CTG) Assay. ATP standard curve was prepared with known concentrations (1, 0.5, 0.25 and 0.1 µM) in cell culture medium. Cells were incubated in CTG solution while shaking at 150 rpm for 15 min at room temperature. Luminescence from each sample was measured through a plate reader (Biotek, Synergy H1). FDA (5 mg/mL in acetone) and PI (2 mg/mL in PBS) were used to stain live and dead cells respectively. Cells (cell number=50,000) were seeded on each hydrogel in 24 well plate and incubated for 48 h. Subsequently, hydrogels were placed in a petri dish and suspended in 2 mL of PBS. The cells were stained with FDA/PI solutions (60 µL of PI and 10 µL of FDA) in dark for 5 min at room temperature. Hydrogels were rinsed with PBS and examined under fluorescent microscope (Nikon eclipse Ni).

2.8. Statistical analysis

All quantitative data has been obtained as a mean of 5 replicates with $\pm$ SD or otherwise mentioned. Two-way ANOVA analysis followed by Tukey's multiple comparison was performed by using p-value of <0.05 .

3. Results and discussion

3.1. Characterization of synthesized materials

Detailed synthesis steps are well explained in sections 2.2 and 2.3 and schematically represented in **Fig. 2**. 9-AA was synthesized as shown in **Fig. 2-step-1A**. To confirm the successful synthesis of 9-AA, ATR-FTIR, UV-Vis spectroscopy and ^1H NMR analysis was performed. In FTIR spectra (**Fig. 3A**), the sharp band at 3048 cm^{-1} was assigned to aromatic -CH stretch of anthracene, whereas in 9-NA, there were additional absorption bands at 1513 and 1373 cm^{-1} positions, which are attributed to the nitro group [26]. In 9-AA, two bands around 3363 and 1620 cm^{-1} correspond to $-\text{NH}$ stretch. The presence of amine groups in spectra confirm the successful synthesis of 9-AA [27]. UV-Vis spectroscopy was carried out to verify the synthesis of 9-AA further. A series of 4 different absorption peaks between 300 to 400 nm (**Fig. 3B**) correspond to anthracene [28]. In 9-AA spectrum, a shift was observed, and a sharp peak appeared around 265 nm depicts the amine group present in the system. Furthermore, synthesis was reconfirmed through ^1H NMR (**Fig. S1**) and results are in accordance with the literature [29]. TNBS assay was used to quantify the conversion% (**Fig. S2-A**).

Methacrylic anhydride (MA) has extensively been conjugated with alginate to enable its covalent crosslinking [30]. In our study, we synthesized MA-alginate by using excess of MA (**Fig. 2-step-1B**). MA-alginate has free carboxylate groups, which were utilized to conjugate with amine groups of 9-AA through EDC/NHS chemistry (**Fig. 2-step-1C**). Although anthracene is itself nonpolar, however, when it is introduced in highly hydrophilic polysaccharide (anth-MA-alginate), the overall system remains water soluble. FTIR spectrum

of sodium alginate (**Fig. 3C**) clearly displays the broad band at 3270 cm^{-1} , which corresponds to the -OH stretching band. Another band at 1595 cm^{-1} position, is related to asymmetric vibration of carbonyl group [31]. Moreover, MA-alginate shows a characteristic band of C=O of ester group at $\sim 1715\text{ cm}^{-1}$ after MA conjugation with alginate [32]. After conjugation of 9-AA with MA-alginate, the band observed at 1560 cm^{-1} is attributed to C=O of amide-II, whereas the band at 1245 cm^{-1} corresponds to C-N stretch as a result of the reaction of amine groups of anthracene with the carboxylic groups of alginate through carbodiimide chemistry [33, 34]. To investigate the synthesis of anth-MA-alginate further, UV-Vis spectroscopic analysis was carried out (**Fig. 3D**). Virgin sodium alginate and MA-alginate did not show any absorption peak, however, anth-MA-Alginate displayed absorption peaks at 280 and 386 nm. These peaks are attributed to the three fused benzene rings in anthracene structure and correspond to π - π transition. Successful incorporation of 9-AA was also confirmed through TNBS assay (**Fig. S2-B**).

3.2. Reversible swelling of hydrogels

To investigate water uptake and stability of hydrogels, swelling experiment is used as a simple and basic method [35]. Swelling of alginate hydrogel depends on acidic pendant groups (-COOH) present in the main chain of alginate. At low pH, the carboxylic groups of alginate get protonate causing shrinkage of hydrogel and vice versa at high pH [36]. Hydrogel swelling occurs in steps; first, water diffuses into the hydrogel network instigating loosening up of polymer chains upon hydration and then, relaxation causes expansion in hydrogel [37]. Water within hydrogel network can also interact with the gel matrix either through hydrophilic or hydrophobic interactions. When dry hydrogel absorbs water, hydrophilic interactions take place through hydration of polar groups leading to primary bound water. Upon polar groups' hydration, the gel network swells, and hydrophobic groups also get expose to surrounding water. Subsequently, water interacts with non-polar groups through

hydrophobic interactions and is known as hydrophobic or secondary bound water [38]. Moreover, hydrogel can also absorb additional water under the influence of osmotic pressure difference and free water diffuses within hydrogel network. An equilibrium is established between elastic forces and osmotic pressure as explained by Flory and Rehner theory [8]. Both hydrogels exhibited swelling at pH 7.4 and deswelling at acidic pH (**Fig. 4B**). The swelling ratio of anth-MA-alginate hydrogel was slightly higher than that of MA-alginate hydrogel. This is primarily due to the large mesh size (**Fig. S3**), more water uptake and also possibly due to the presence of hydrophobic moieties (anthracene) which lead to the absorption of additional water through secondary bound water interactions. A visual demonstration was also performed and alteration in hydrogel size was measured after incubation in different pH solutions at specific time points (**Fig. 4A**). We prepared hydrogels as circular disks (0.9 ± 0.05 cm). MA-alginate hydrogel was clear and transparent, while anth-MA-alginate hydrogel appeared as slightly yellowish and opaque due to the presence of 9-AA within hydrogel structure. In acidic pH (2.2), both hydrogels collapsed, and their size reduced to 0.6 ± 0.1 and 0.8 ± 0.1 cm for MA-alginate and anth-MA-alginate, respectively. However, at physiological pH (7.4), both hydrogels exhibited swelling and the size of both hydrogels was increased to ~ 1 cm.

3.3. Drug loading and release assessment

For drug loading and release studies, we loaded DOX into MA-alginate and anth-MA-alginate hydrogels. Drug loading efficiency was found to be $85.3 \pm 1.8\%$ and $96.1 \pm 0.4\%$ for MA-alginate and anth-MA-alginate hydrogels, respectively. Higher loading efficiency observed for anth-MA-alginate hydrogel is possibly due to the large mesh size and the interaction of DOX with anthracene groups conjugated to hydrogel. The percent DOX release profiles from MA-alginate and anth-MA-alginate hydrogels after 144 h, at physiological pH (7.4) were measured as $\sim 30\%$ and $\sim 20\%$, respectively (**Fig. 4C and 4D**). When these hydrogels were

incubated at pH 5, percent DOX release from MA-alginate and anth-MA-alginate hydrogels were increased significantly to ~60% and ~55%, respectively. Cumulative drug release was also measured in more acidic solution (pH 2.2) in order to reconfirm the pH dependent release profile and the rates were drastically increased to ~95% and ~85% for MA-alginate and anth-MA-alginate hydrogels, respectively. These observations verify that the total amount of DOX release is directly influenced by the pH of the medium. For example, after 24 h, the amount of DOX released from MA-alginate hydrogels was ~80%, ~40% and ~25% at pH 2.2, 5.0 and 7.4, respectively (**Figure 4C**). On the other hand, anth-MA-alginate hydrogels resulted in comparatively lower amount of release which was around ~75%, 35% and 16% at pH 2.2, 5.0 and 7.4, respectively (**Figure 4D**). Entrapped molecules in a hydrogel network can release through different ways. Diffusion-controlled release mechanism is common in a system that can swell and the release follows Fick's law of diffusion [8]. Furthermore, the release pattern is also dependent on the solubility of drug in different pH buffers. DOX is highly soluble at lower pH which may augment its fast release from the hydrogel due to the protonation of amine groups present on DOX [39]. However, at pH 7.4, carboxylate of alginate carries a negative charge and amino groups of DOX exhibit a positive charge causing electrostatic interaction between the drug and the network. Hence drug molecules get entrapped and release negligibly at high pH and vice-versa [40]. Furthermore, we have observed that DOX release from anth-MA-alginate hydrogel is slightly lower than the release observed from MA-alginate. This can be attributed to the similarity of DOX structure with anthracene [41, 42] and hence its hydrophobic interaction with the network which in turn hinders its fast release compared to MA-alginate hydrogel. These results show that our system is somehow dependent on pH sensitivity of the hydrogel and anthracene incorporated hydrogel presents more sustained release.

3.4. Rheological studies

To investigate the mechanical properties of MA-alginate and dual crosslinking of anth-MA-alginate hydrogels, rheological analysis was performed using rheometer. The storage modulus increased rapidly for both gels due to the formation of effective elastic intermolecular crosslinks (**Fig. 5A**). It increased sharply within the first 65 seconds, and then continued to increase until 350 seconds. G' of MA-alginate was leveled off after ~500 seconds which indicated the completion of photocrosslinking. However, in case of anth-MA-alginate, photocrosslinking continued until ~1000 seconds. Slightly improved modulus observed for anth-MA-alginate can be ascribed to photodimerization of anthracene. The contribution of photodimerization to higher modulus was further verified through gelation with altered concentration of 1, 1.5 and 2% (w/v) anth-MA-alginate solutions without the use of any photoinitiator, coinitiator and accelerator (**Fig. 5B**). Increase in modulus can be observed with increasing anthracene concentration, verifying enriched crosslink density due to photodimerization of anthracene. Gel made through only photodimerization of anthracene was also robust and mechanically stable except for the 1% (w/v) anth-MA-alginate solution. For the gels prepared with 1% and 1.5% (w/v) polymer solutions, distinctive gel point could not be observed due to smaller number of photodimerizable moieties present in the system. However, for 2% (w/v) polymer solution, the gel point was clearly observed around 300 seconds. Mechanically robust gels fabricated through photodimerization only, can have great significance due to the absence of toxic materials such as photoinitiator and coinitiator etc. As dedimerization of anthracene happens at ~254 nm, therefore, cyclic dimerization and dedimerization can be carried out at 365 and 254 nm depending on the need. This phenomenon was demonstrated repeatedly to show sol-gel transition and vice versa (**Fig. 5C**). The photodimerization of anthracene is based on Diels-Alder cycloaddition reaction in which, $[4\pi + 4\pi]$ cycloaddition reactions are involved between two anthracene molecules [43]. The $[4+4]$ photocyclodimerization is usually obtained by exposing anthracene containing

molecule to UV light (365nm) and its dedimerization takes place at 254nm (**Fig. 5D**). After dimerization, anthracene is unable to absorb further light at wavelength between 300 and 400 nm, which is a facile way to monitor the completion of dimerization reaction spectrophotometrically.

3.5. Cell viability analysis

Cytocompatibility of MA-alginate and anth-MA-alginate hydrogels was investigated through cell viability assay (**Fig. 6**). Percent survival of HeLa cells in MA-alginate hydrogel was measured as ~86% on day-1 and ~96% on day-4. Slightly lower viability on day-1 might be due to the anticancer property of alginate as observed in previous studies [44]. On the other hand, for anth-MA-alginate hydrogel, viability of HeLa cells significantly reduced to ~65% (** $p < 0.01$ for day-1 and **** $p < 0.0001$ for day-4) (**Fig. 6A**). The compromised HeLa cell proliferation and hence lower viability is probably due to the presence of anthracene derivative within our hydrogel structure. To check the impact of anthracene on healthy cells, we also investigated the viability of NIH-3T3 fibroblast cells. Percent cell survival observed for NIH-3T3 fibroblast cells in MA-alginate and anth-MA-alginate hydrogels was >95% and ~85% on day-1, respectively (**Fig. 6B**). Similarly, both kind of hydrogels did not compromise the proliferation rate of fibroblast cells on day 4, where viability measured was >100% compared to the control group. These observations suggest that presence of anthracene derivative compromised the growth of cancer cells and endorse our hypothesis. In our previous study, PEG hydrogel coated nanocarriers were loaded with DOX, where the effect of DOX release from enzymatically degradable network influenced fibroblast and HeLa cell viability [45]. HeLa cells were strongly affected than healthy fibroblast cells, probably due to the fewer number of integrins in case of fibroblast cells. Cell seeded hydrogels were further visualized through Live/Dead assay (FDA/PI staining) under fluorescence microscope (**Fig. 6C-6F**). Predetermined number of fibroblast and HeLa cells suspended in medium were

seeded on hydrogels. The green color represents live cells (FDA stained) while dead cells express red color (PI staining). Fibroblast cells showed good viability on both types of hydrogels and most of the cells were alive even after 48 h (**Fig. 6C and 6D**). On the other hand, HeLa cells exhibited a compromised growth on MA-alginate in general and particularly on anth-MA-alginate hydrogel (**Fig. 6E and 6F**) based dead cells observed. These results are also consistent with our cell viability assay. Cells demonstrated spherical morphology due to the lack of integrin engagement sites available in alginate to sense mechanical stiffness within the microenvironment [20, 46, 47]. According to a previous study, NIH-3T3 cells are very prone to form spherical morphology if they do not get a suitable place for adhesion and due to the spongy nature of hydrogel, can form aggregates as well. [48]. Cell-aggregation in HeLa cells is also a reported phenomenon but mechanism is unknown [49]. Besides many possible explanations regarding aggregate formation, porosity of hydrogel might be a significant reason. Pores in a 3D hydrogel may allow cell migration and so interaction with neighboring cells happens depending on their physical locations and the secretory molecules surrounding them. Further, crosstalk permitting enhances ECM protein synthesis, which in turn can promote cell aggregation [50-52]. Consequently, few cells remain on gel surface and rest of the cells migrate towards the interior domains within the hydrogel through the pores and finally form spheres [49].

4. Conclusions

Preparation of unique pH-responsive anthracene-modified alginate-based hydrogel for selective release of doxorubicin as cancer therapeutics has been reported. Impact of anthracene conjugation on physical, mechanical and biological properties of hydrogel is critically evaluated. Incorporation of anthracene promoted porosity, swelling, and mechanical properties of photopolymerized hydrogel due to photodimerization of anthracene. It also led towards reversible control on crosslinking as evidenced by transition between gel/sol states

through dimerization and dedimerization of anthracene groups at 365 nm and 254 nm, respectively. Moreover, these hydrogels resulted in sustained and increased DOX release at acidic pH mimicking the cancer tissue microenvironment. Cell viability analysis and live/dead assay revealed that growth of HeLa cell was comparatively compromised on anthracene conjugated alginate hydrogels with little or no effect on healthy cells. Conclusively, we can say that, anth-MA-alginate hydrogel with reversible crosslinking behavior can be a promising candidate for targeted cancer therapeutics. All these invaluable characteristics may lead towards the design of a smart system requiring low dose of antitumor drug for cancer therapeutics.

Appendix:

Supplementary data file.

Credit authorship contribution statement:

Syeda Rubab Batool: Methodology, Investigation, Writing-original draft. **Muhammad**

Anwaar Nazeer: Conceptualization, Methodology, Investigation, Writing - review & editing.

Duygu Ekinci: Methodology, Writing - review & editing. **Afsun Sahin:** Writing - review &

editing, **Seda Kizilel:** Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Acknowledgements:

This study is supported by the Scientific and Technological Research Council of Turkey (TUBITAK) under International Support Program (COST Action - European Cooperation in Science and Technology - CA18132, project number: 119M171) and partially by Koç University Seed Fund SF.0028. The authors gratefully acknowledge the use of facilities of Koç University Research Center for Surface Science (KUYTAM) and Koç University Research Center for Translational Medicine (KUTTAM), funded by the Presidency of Turkey, Presidency of Strategy and Budget. The content is solely the responsibility of the

authors and does not necessarily represent the official views of the Presidency of Strategy and Budget.

Declarations of interest: None

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International journal of biological macromolecules, Batool et. al, Fig. 1.

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International journal of biological macromolecules, Batool et. al, Fig. 6.

*Color should be used for all figures in print.

Figure captions:

Fig. 1. Anth-MA-alginate hydrogel formation through photopolymerization ($\lambda=514$ nm) and photodimerization ($\lambda=365$ nm).

Fig. 2. Key steps involved in materials synthesis and hydrogel formation

Fig. 3. (A) FTIR and (B) UV-Vis spectra of anthracene, 9-NA and 9-AA. Characterization of alginate, MA-alginate and anth-MA-alginate through (C) FTIR and (D) UV-Vis

Fig. 4. (A) Optical images and (B) cyclic pH responsive behavior of hydrogels at acidic and physiological pH. Cumulative drug release% for (C) MA-alginate and (D) anth-MA-alginate hydrogels at different pH conditions.

Fig. 5. Storage (G') and loss modulus (G'') as a function of time for (A) photopolymerized MA-alginate and anth-MA-alginate hydrogels (with photoinitiator) and (B) photodimerized anth-MA-alginate (without photoinitiator). (C) Visual and (D) schematic demonstration of dimerization/dedimerization behavior of anth-MA-alginate in the absence of photoinitiator.

Fig. 6. Cell viability analysis of hydrogels against (A) HeLa and (B) NIH-3T3 cells (**** $p<0.0001$ and ** $p<0.01$). Representative fluorescence images of live/dead cells (FDA, green/PI, red) after 48h of seeding for NIH-3T3 cells on (C) MA-alginate, (D) anth-MA-alginate (D), and HeLa cells on (E) MA-alginate and (F) anth-MA-alginate hydrogel.

Graphical abstract

HIGHLIGHTS

- Anthracene functionalized MA-alginate was obtained through 9-Aminoanthracene
- Dual crosslinking was performed through photopolymerization and photodimerization
- Enhanced storage modulus was observed for dual crosslinked hydrogels
- Fabricated hydrogels exhibited pH dependent release of doxorubicin
- Anthracene compromised cancer cell growth with no effect on healthy cells