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Interactions governing the entrapment of anticancer drugs by low molecular weight hydrogelator for drug delivery applications

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We present the effect of size, charge, and hydrophobicity of anticancer drugs on their drug encapsulation efficacy in *L*-Alanine based small molecule hydrogelator. Entrapment of various anticancer drugs in hydrogel were depicted and were correlated towards interaction between gelator and drug molecules. Hydrogel showed highest entrapment for 5-Fluorouracil as high as ~1.2 mg/mL in 1.5 % (w/v) hydrogel; whereas with small polar anticancer drugs like Cisplatin and Carboplatin, poor encapsulation was observed. Hydrogel was also able to entrap and retain the hydrophobic drugs like Docetaxel and Tamoxifen with a high drug loading efficiency. The drug entrapped hydrogels were then characterized by rheology and SEM studies to understand the effect of drug on hydrogel assembly. Drug release and anticancer activity studies showed slow and sustained release of drugs from hydrogels, making them suitable for exploring in direction of future cancer therapeutic applications.

Introduction

Current cancer chemotherapy regimes include use of biomaterials based on polymers, liposomes, or protein based drug nanoparticles that help in controlled release of drugs in blood circulation for enhanced bio-distribution and improved pharmacokinetics.[1] These cancer chemotherapeutic approaches have strong limitations such as several systemic toxic effects and poor targeting at tumor sites[2] that calls for improved localized injectable therapies that can inevitably surpass such limitations by enhancing localized drug concentrations and minimizing systemic toxicity coupled with reduced dosage through slow and sustained release.[3]

Hydrogels provide suitable alternatives to existing clinically available biomaterials due to their ability to encapsulate and allowing slow and sustained release of drugs.[4] Injectability of these hydrogels at tumor sites provide additional benefits of releasing drugs at tumor sites and avoiding systemic toxicity of drugs.[5] In this regard, low molecular weight hydrogelators (LMHGs) gained major attraction due to their small molecular weight and their ability to self-assemble into mechanically robust gels that can easily encapsulate drugs and subsequently release them in a sustained manner.[6] Many LMHGs based on peptides[7], semi-synthetic molecules[8], carbohydrates[9] or lipid based[10] carriers have been reported. Unlike the conventional polymeric carriers, these LMHGs (M.W. <1000 Da) can be easily synthesized and have known biodegradable pathways causing less side-effects.[11] These LMHGs basically dwell upon secondary interactions such as hydrogen bonds, Van der waals forces, π-π stacking, and electrostatic interactions rendering their self-assemblies to be structurally reversible.[12] These interactions also help the hydrogels in retaining different drugs of variable charge and hydrophobicity.[13] Sutton *et al.* showed gelation behavior of Fmoc-phenylalanine and Fmoc-tyrosine gels for a pH sensitive Fickian drug release.[14] Banerjee and co-workers have developed many peptide based gelators and explored their thixotropic behavior, drug encapsulation and release studies.[15] A peptide amphiphilic system for Cisplatin delivery was developed where peptide molecule having MMP-2 sensitive sequence GTAGLIGQRGDS was used.[16]

As majority of the commercial anti-cancer drugs are highly hydrophobic, it calls for an imminent need to design hydrogels that can entrap higher amounts of such drugs. In many instances, LMHGs are amphiphilic[17] where gel formation is assisted by both H-bonding as well as hydrophobic interactions. Though gels formed out of hydrophobic molecules possess higher strength, very few systems have been explored so far for their biomedical utility.[18] The challenges in homogenous dispersion of gelator molecules in water prior to gelation limits the homogenous supramolecular assembly and thereby gelation. Gao *et al.* made first attempts in forming hydrogels using hydrophobic interactions, where gelation was induced by phosphatase assisted flipping of hydrophilic precursor to hydrophobic molecule.[19] Using similar strategy, Yang *et al.* developed Taxol-Folic acid derivative that underwent self-assembly by coupling with a motif GpYk

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followed by a phosphatase catalysed reaction.[20] Cheetham *et al.* adopted a unique approach by developing structures using assembly of anticancer drug, Camptothecin itself.[21]

Though hydrogelator-drug conjugates display a clear advantage in terms of slower and controlled drug release, the need of reactive functional groups to form reversible linkages during conjugation limits the drug conjugation strategy to lower number of molecules. Moreover, the conjugation always involves tedious synthetic procedures that can also be unfruitful in terms of cost effectiveness and accessibility to non-specialists. Therefore, physical entrapment of drug molecules directly in LMHG hydrogels still stands as a sound and popular strategy for entrapping drugs. It offers the advantage of entrapping a wide range of payload molecules individually or in entrapping combination of multiple drugs – of special relevance in cancer therapy.[22]

Recently, we reported injectabile hydrogels prepared from an *L*-Alanine derivative (ALA-HYD) that could encapsulate and release Doxorubicin, a potent cancer chemotherapeutic drug. Significant reduction in the tumor volumes were observed when drug entrapped hydrogel was injected at tumor site in mice.[23] Formed at an optimum gelator concentration of 1.5 % (w/v), these hydrogen bonded based hydrogels exhibited fairly good mechanical strength and thixotropic behaviour. As anticancer drugs vary from each other in terms of their aqueous solubility, hydrophobicity and charge, it is impossible to develop a universal hydrogelator system for all kinds of anticancer drugs. To best of our knowledge, no study has been performed to systematically investigate the therapeutic potential of different anticancer drugs getting encapsulated in a given LMHG.[24]

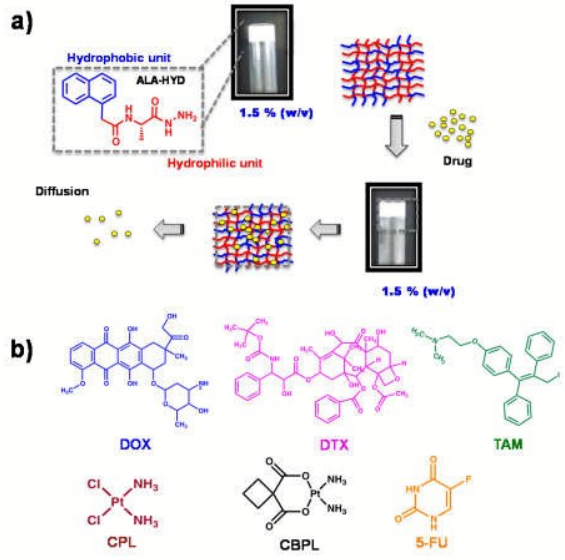

Figure 1. a) Schematic outline of present study depicting drug encapsulation in *L*-alanine based hydrogelator, ALA-HYD, having both hydrophillic and hydrophobic moieties in its fibrillar network and its release by diffusion; b) Chemical structures of six different anticancer drugs Doxorubicin (DOX), Docetaxel (DTX), Tamoxifen (TAM), Cisplatin (CPL), Carboplatin (CBPL) and 5-Fluorouracil (5-FU) used in this study.

Therefore, to address this issue we studied encapsulation efficacy, drug release, mechanical strength, injectability, and anticancer potential of ALA-HYD hydrogel using six anticancer drugs differing in charge and hydrophobicity *viz*. Docetaxel (DTX), Tamoxifen (TAM), Cisplatin (CPL), Carboplatin (CBPL) and 5-Fluorouracil (5-FU) along with Doxorubicin (DOX) (Figure 1). Each drug has a different mode of action towards cancer cells, as DTX is known for its activity by inhibiting the micro-tubule depolymerization[25] and TAM acts as anti-estrogen in mammary tissues.[26] The platinum drugs, CPL and CBPL both bind with DNA to form intra-strand crosslinks thus affecting replication,[27] whereas 5-FU generally inhibits nucleotide synthetic enzyme thymidylate synthase.[28] In this manuscript, the drug encapsulation efficiency, release and characteristic anti-cancer potency of ALA-HYD gelator for different anticancer drugs differing in their size, hydrophobicity, H-bonding ability were investigated.

Results and discussion

The initial studies started with determining the encapsulation efficacy of 1.5% ALA-HYD hydrogel for different anticancer drugs. Measured amounts of drug stock solutions were added to hot aqueous super saturated solution (sol) of gelator and addition was continued till integrity of gel was retained. Figure 2 shows inverted vial images of hydrogel formation entrapping different drug molecules, where ability of gelator to gelate solvent, decreased with increasing amount of drug added to sol prior to gelation. Encapsulation efficacies differed with each drug, with least entrapment achieved with Cisplatin (80 µg) and highest (640 µg) amount of entrapment with 5-FU under these conditions (Table 1).

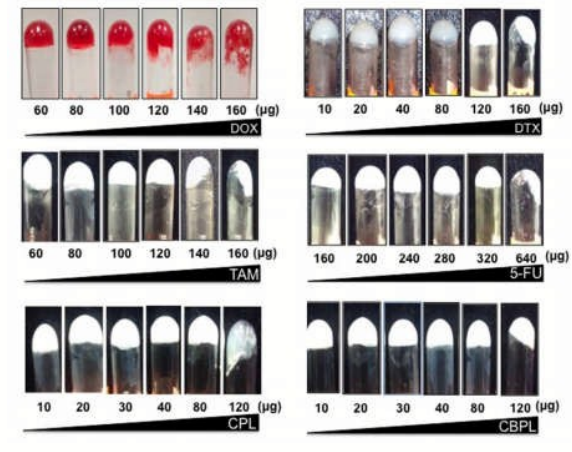

Figure 2. Digital images of Inverted vials with hydrogels showing gelation and maximum encapsulation efficiency w.r.t to drugs DOX , DTX , TAM, 5-FU, CPL and CBPL. Encapsulation efficiency is indicated as amount of drug (µg) per 500 µL of 1.5 %(w/v) ALA-HYD hydrogel..

Differential encapsulation efficacies for anticancer drugs might be due to differential non-covalent inter- and intra-molecular interactions such as hydrogen bonding, van der Waals and π-π stacking between drug and gelator molecules. Self-assembly is a prevalent phenomenon that leads to gelation for LMHGs in absence of chemical cross-linkers. Therefore, significant differences in encapsulation efficacy can be directly correlated with degrees of hydrophobicity and molecular structures of drugs. 5-FU being smallest moiety and having somewhat planar architecture has ability to get encapsulated the most. Presence of amide bonds might provide hydrogen bonded water solubility whereas fluorine group provides weak hydrophobic interactions. A balance of these two forces along with π-π stacking as another possible intermolecular interaction between drug, solvent and gelator led to gelation and high drug encapsulation. Further, due to its small size, 5-FU is expected to cause least perturbation to the gelator assembly, leading to stable gels even at high drug loadings.

Table 1. Maximum encapsulation efficacy of ALA-HYD for different anticancer drugs and their injectability.

Drug	Maximum Encapsulation (μg/500 μL)	Maximum Injectability (μg/500 μL)
Cisplatin (CPL)	80	80
Carboplatin (CBPL)	120	80
Docetaxel (DTX)	160	80
Doxorubicin (DOX)	200	120
Tamoxifen (TAM)	240	120
5-Fluorouracil (5-FU)	640	640

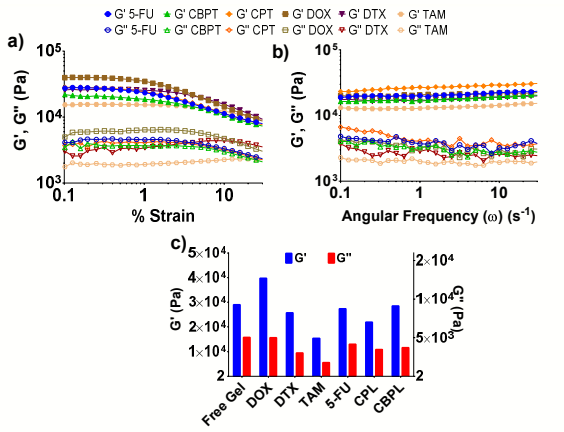

Figure 3. Rheological studies showing a) Frequency sweep, b) Amplitude Sweep, and c) Storage modulus (G') variation in the free gel and drug loaded gels tested at a capacity of 80 μg/ 500 μL of gel volume. These studies point out mechanical stability of gels upon entrapment of (G' > G'') and their varied strengths as an outcome of the gel-drug interactions with their strengths varying with drug entrapped.

Order of encapsulation among DOX, DTX, and TAM anticancer drugs was TAM > DOX > DTX (Figure 2, Table 1); as TAM presents maximum aromatic planar architecture for aromatic interactions between itself and the gelator molecule, whereas DTX is highly hydrophobic in nature with minimum ability for π-π interactions. Very surprisingly, hydrogel showed minimum encapsulation efficacy for CPL and CBPL in spite of small size and polar nature of these drugs, suggesting that presence of free amine groups on CPL and CBPL disrupt the H-bonding between gelator molecules at high concentrations. Absence of any aromatic interactions between these molecules and ALA-

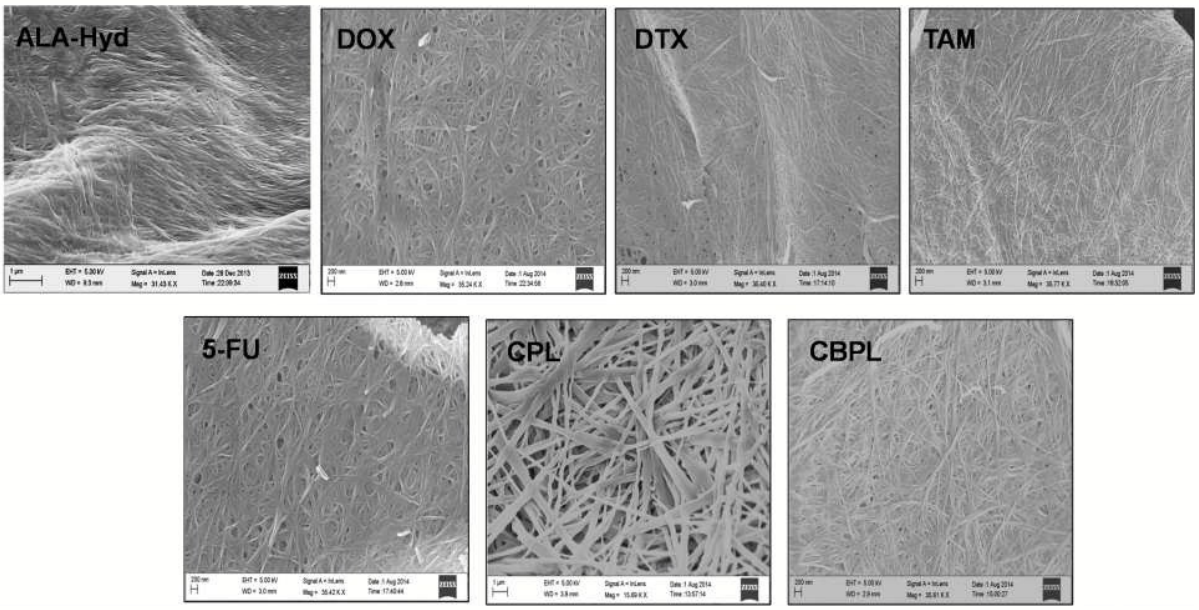

Figure 4. Scanning electron micrographs of gels with different drugs at 80 μg encapsulation. Scale bar is 200 nm for all the gels except CPL that is shown at a scale of 1 μm.

HYD further alleviates the problem of their getting encapsulated in the hydrogel.

Effect of the drug entrapment on injectability of hydrogels having different anticancer drugs was also checked simultaneously. Injectability of gel followed a regressive pattern with increase in concentration of drug entrapped with only exception of 5-FU where gel was injectable even at its maximum encapsulation. A maximum of 80 µg of each drug could be incorporated into gel (per 500 µL) while maintaining injectability. We then performed rheological studies including both amplitude sweep and frequency sweep experiments on drug encapsulated hydrogels as shown in Figure 3

The *G*′ (storage modulus) and *G*′′ (loss modulus) values clearly indicate formation of mechanically stable gels (*G*′ > *G*′′). Hydrogels entrapping DOX had highest mechanical strength, with *G*′ value even higher than pristine gel that might be due to formation of imine bond between DOX and the gelator.[23] However, TAM-entrapped gel exhibited lowest *G*′ amongst the systems tested, and was almost half of the pristine gel. Lower mechanical strength of Tam-entrapped gel might be due to presence of tertiary ammonium unit in the drug interfering with self-assembly of gelator, resulting in a significant decrease in gel strength. It is noteworthy that self-assembly of gelator is resilient enough to accommodate a large hydrophobic molecule like DTX without significant decrease in gel-strength. Not surprisingly, gels could readily accommodate 5-FU without much difficulty, and resulting gels were as strong as the pristine ones. CBPL entrapped gel was mechanically less stable compared to CPL entrapped gel due to molecular complexity of CBPL over CPL, and inability of carboxylates over chloride ligands in getting accommodated in gelator molecules.

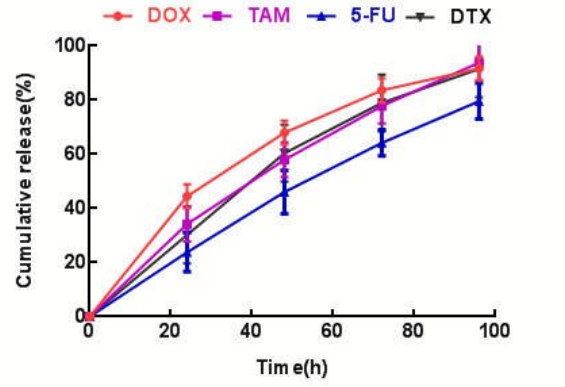

Figure 5. Cumulative drug release profiles from hydrogels entrapping 80 µg of DOX, DTX, TAM and 5-FU respectively over a course of 96 h showing the DOX being released at a faster rate and 5-FU with slow and consistent release compared to all other drugs.

SEM studies indicated that there are not much perceptible differences in the nanoscale morphology of hydrogels except in CPL entrapped gel (Figure 4). CPL-entrapped gel showed hollow rod-like structures whereas with other drug-encapsulated gels showed long entangled fibrous morphology similar to pristine gels. Higher hydrogen bonding ability of $-NH_2$ moieties of CPL drug with gelator molecules and higher planarity of drug facilitates its easy incorporation into intra-fibrillar network leading to altered morphology of gel. In contrast non-planarity of CBPL diminshes gel-drug interactions and we find no perceivable change in architecture with CBPL-entrapped gel.

We then performed drug release studies of these hydrogels at 37 °C (Figure 5) at physiological pH. DOX loaded hydrogels showed the maximum and fastest release where 80% of the drug was released over 96 h, whereas 5-FU hydrogels exhibited slowest release with around 70% release in 96 h. 5-FU showed maximum incorporation into the gel being a structurally smaller moiety as compared to the other drugs (Table 1) and such a slow and sustained release pattern suggested favorable interactions and compatibility between 5-FU and gelator molecule. Interestingly, mechanical strength of 5-FU loaded gels was also comparable with that of free gel. DTX and TAM loaded hydrogels showed a similar trend with 60% release after 48 h. We could not, however, study the release studies of CPL and CBPL loaded gels as it was difficult to detect the released amounts with HPLC (being below the detection limit of the instrument). This could be due to strong interactions between $-NH_2$ groups of drugs and gelator molecules, which might have led to a much slower release which can also be accounted for their least incorporation into the gel structure as compared to the other four drugs.

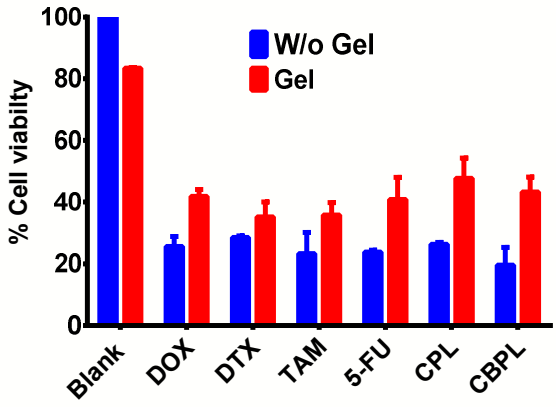

Figure 6. Cytotoxicity towards 4T1 cell lines of free drug as well as drug entrapped in hydrogels indicating reduced toxicity of the drugs entrapped in hydrogel, in comparison to those added directly to the cells, owing to the controlled release of drug from the gel network. Mean ± SD, n = 3.

The gel network provides a sustained release of drugs which help the drugs to retain at the tumor site for longer time thereby increasing their anti-cancer potency for a longer time periods. The sustained release also helps in preventing the recurrence of tumor. The anticancer potential of drug encapsulated hydrogels against murine breast cancer 4T1 cell line was investigated. 4T1 cells were cultured on lower compartment of Trans-well plate and drug loaded hybrid gels were cast on Trans-well. We performed MTT assay to study cell proliferation in 4T1 cells in presence of free drugs and also the drugs entrapped in gel. Highly entrapped drugs 5-FU, CPL

and CBPL showed almost a fold difference in the cell viabilities of gel entrapped and free drug. Comparison of cell death between drug and hydrogel-drug treated cells (Figure 6) suggested that drug loaded hydrogels are less toxic towards the cells as compared to those treated with free drugs that further proves the controlled release of the drugs from the hydrogel.

Conclusions

In summary, we studied ability of *L*-Alanine derived hydrogelator ALA-HYD to physically entrap various anticancer drugs. ALA-HYD could entrap all the drugs with different capacity by mere physical mixing without use of any covalent or coupling strategies. All six drugs formed injectable hydrogel systems at different loading capacities indicating optimum mechanical strength. These gel-drug systems revealed the nano-fibrous morphology and potential to show therapeutic applicability. CPL and CBPL showed least incorporation into gel network owing to their ability to form stronger interaction with gel molecules, thereby disrupting physical interactions among gelator molecules. *In vitro* release of drugs *via* process of diffusion at 37 °C showed desirable slow and sustained release of drugs in each case that was evident from an increased cell survival as compared to free DOX and 5-FU. This system, therefore, further opens up possibilities for dual encapsulation of drugs in several combinations (hydrophilic-hydrophilic or hydrophilic-hydrophobic) and testing their efficacy against tumors. Present study not only introduces a suitable carrier molecule for hydrophobic drugs but increases the scope of further encapsulating a suitable combination of two drugs in the same gel so as to increase its efficiency.

Experimental Section

Materials and methods: Anticancer drugs Doxorubicin, Docetaxel, Tamoxifen, 5-Fluorouracil, Cisplatin and Carboplatin; and Sodium Dodecyl Sulphate (SDS), Tween-80 were purchased from Sigma Aldrich. HPLC grade Methanol and Acetonitrile were from Spectrochem. Gelator compound ALA-HYD was synthesized as described earlier.[23]

Preparation of hydrogel: The hydrogels was prepared with a gelator concentration of 1.5 % (w/v) in aqueous medium with the method reported earlier.[23] Gelation was checked by inverting the glass vials.

Drug encapsulation and injectability studies: Drug stocks were prepared in their respective solvents, like hydrophilic drugs namely DOX and 5-FU were dissolved in water whereas hydrophobic drugs like TAM and DTX was dissolved in DMSO. Methanol was used for CPL and CBPL. From these drug stocks, desired amounts of drugs were added to gel solution to find out 'maximum encapsulation capacity' and 'maximum injectability limit for these drugs individually. 'Maximum encapsulation capacity' is simply the maximum amount of drug that could be taken up by the gel without disrupting the structure while 'maximum injectability' limit was the maximum amount of drug that can be encapsulated in the gel while retaining the injectability. Inverted vial method was used to demonstrate gelation that was further confirmed by Rheology. Gelation was checked minimum three times for each drug:gel ratio. Injectability was tested using a surgical 20" needle generally used for *in vivo* experiments. As a control, gel was prepared by adding DMSO of volume equal to highest amount of drug solution added to ensure the loss of gel integrity is solely due to the drug but not due to the DMSO.

Rheology studies: For the Strain Sweep experiments, 25 mm diameter and 1˚ angle cone was used at top and flat plate was used at bottom. Samples were placed on the bottom plate. 50 µm gap distance were maintained between the cone and the plate. All the rheological studies were performed at 25 ˚C on the drug entrapped hydrogels (80 µg and at injectability limit of drugs in 15 mg/mL gelator). Strain-sweep tests were performed from 0.01 to 100% strain at constant frequency = 1 $rad.s^{-1}$. Frequency sweep experiments were carried out from 0.1 to 100 Hz at a constant strain of 0.1%.

Scanning electron microscopy (SEM): Drug entrapping hydrogels (80 µg of drugs at their injectability limit in 15 mg/mL gelator) were dried inside vacuum desiccator for 60 h. These dried samples were spread on carbon tape and gold coated for 120 sec. The images were taken at 5 kV accelerating voltage on Carl Zeiss (Ultraplus) Field emission scanning electron microscope.

Drug release studies: We initially prepared drug release media for each drug for carrying out in-vitro release assay. Hydrophilic drugs were tested in phosphate buffer saline (PBS) alone with a pH of 7.4. For DTX, 0.01% Tween-80 was added to the buffer to enhance its solubility, whereas for TAM, 0.1% SDS was mixed with PBS in a ratio of 14:1 v/v. The drug-loaded hydrogels were immersed in their respective release media and aliquots were taken out at stipulated time intervals of 24, 48, 72 and 96 h followed by replacement with fresh media. For each drug, the release assay was carried out three times.

High-pressure liquid chromatography (HPLC) studies: Released drugs were quantified using HPLC with the help of a standard curve using Waters HPLC (Germany) equipped with a UV/Visible detector and TSK gel ODS 100V 5µm column. For all the systems, HPLC Acetonitrile, Methanol, Milli Q double filtered water was used as the mobile phases. UV detection at respective wavelengths was carried out in each case.

In vitro cytotoxicity (MTT assay) studies: Mouse mammary gland cell line (4T1) was cultured in RPMI 1640 medium (Sigma-Aldrich), supplemented with 10% heat-inactivated fetal calf serum (FCS; Invitrogen), 1% (v/v) Penn-Strep, at 37°C, under 5% CO_2 in a humidified atmosphere. We seeded ~1.0×10^4 cells per well in lower compartment of a 24 well corning trans-well plate. Cells were seeded 1 day before the experiment for proper attachment, and treated with various anticancer drugs at concentrations near to their IC_{50} values reported in the literature.[30] (DOX- 0.25 µg/mL; 5-FU- 2.3 µg/mL; CPL- 2.8 µg/mL, CBPL- 36 µg/mL, DTX- 0.6µg/mL and TAM- 6µg/mL) and drug entrapped in hydrogels (same concentrations) casted in trans-well filters. MTT assay was performed after 48h. The % cell viability was reported by

comparing the absorbance of Blank cells (w/o gel) as 100% viability.

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