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Biopolymer-Connected Liposome Networks as Injectable Biomaterials Capable of Sustained Local Drug Delivery

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Abstract

Biopolymers bearing hydrophobic side-chains, such as hydrophobically modified chitosan (hmC), can connect liposomes into a gel network via hydrophobic interactions. In this paper, we show that such liposome gels possess an attractive combination of properties for certain drug delivery applications. Their shear-thinning property allows these gels to be injected at a particular site, while their gel-like nature at rest ensures that the material will remain localized at that site. Moreover, drugs can be encapsulated in the interior of the liposomes and delivered at the local site for an extended period of time. The presence of two transport resistances – from the liposomal bilayer and the gel network – is shown to be responsible for the sustained release; in turn, disruption of the liposomes both weakens the gel and causes a faster release. We have monitored release kinetics from liposome gels of a cationic anti-cancer drug doxorubicin (Dox) encapsulated in liposomes. Sustained release of Dox from these gels and the concomitant cytotoxic effect could be observed for over a week.

Keywords

Liposome; injectable gel; chitosan; drug release; doxorubicin

Introduction

Liposomes or vesicles are biomimetic containers formed from natural or synthetic lipids. These structures are known for their ability to encapsulate hydrophilic moieties within their aqueous core, as well as hydrophobic moieties within their bilayer shell.^{1, 2} The encapsulated chemicals will tend to slowly leak out through the bilayer into the external fluid, thereby allowing liposomes to serve as convenient delivery agents for sustained

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Supporting Information

Dynamic rheology of Dox-lipo liposome gel (Figure S1), steady-shear rheology data on Dox-lipo liposome gel (Figure S2), and residual Dox concentration for cell viability test (Figure S3) are presented. This material is available free of charge via the Internet at <http://pubs.acs.org>.

release.^{1, 3–9} Another class of popular materials for sustained release are polymer hydrogels, which are formed by cross-linking polymer chains in water via chemical or physical bonds.^{10–14} These gels are frequently used to deliver encapsulated therapeutic molecules such as drugs, vaccines or peptides.

Over the last two decades, several researchers have attempted to combine the properties of liposomes and polymer gels within the same material.^{15–20} Such hybrids have some attractive features, including the fact that liposomes in a gel can be more stable to environmental stimuli compared to bare liposomes in solution. Moreover, when a drug is placed inside the liposomal core and the liposomes are included in a gel network, the drug will experience a combination of transport resistances due to the liposomal bilayer and the network itself – in turn, this can ensure release of drug over a longer period of time. It can also avoid the problem of “burst release” seen with some polymer gels where a large bolus of drug is released initially from the gel, which can cause toxicity.

The simplest way of combining liposomes and polymer gels is to embed the liposomes in the polymer gel matrix. In this case, there are no active connections between the polymer gel strands and the liposomes. The first such study was performed by Weiner et al. who embedded liposomes in a collagen matrix.¹⁵ They found that their hybrid gel prolonged the retention time of encapsulated hormones. Following this study, liposomes have been embedded in gels of various kinds, including gels of biopolymers such as xanthan gum, gelatin, chitosan, and carboxymethylcellulose, as well as gels of synthetic polymers such as polyacrylic acid.^{15–20} Such hybrid gels could be used in applications that require implantable biomaterials; however, most of these systems are not suitable for introduction by injection because the materials are too rigid and elastic. For gels to be injectable, they must exist in a low-viscosity form prior to or during injection, and they must rapidly set into an elastic gel following injection. Injectable biomaterials are attractive for site-specific drug delivery.^{21–23} For example, injection of a gel containing a cytotoxic anti-cancer agent to the site of a tumor can serve to kill the tumor while ensuring that the majority of drug remains localized near the tumor site and never reaches high levels in any other part of the body. Ideally, such release should be sustainable for an extended time period, i.e., many days, so that multiple repeated injections can be minimized.

Recently, we have studied a different kind of liposomal gel where the liposomes are actively connected to the gel matrix via polymer chains.²⁴ The polymer used was a hydrophobically modified chitosan (hmC) where the chitosan backbone was decorated with a small number of hydrophobic (n-dodecyl) side-chains (schematic in Figure 1). When hmC was combined with liposomes, the hydrophobes from the polymer embedded themselves within liposomal bilayers due to hydrophobic interactions. Thereby the hmC chains connected the liposomes into a network, with the liposomes being the nodes or cross-link points in such a network.

In the present study, we explore the potential for hmC-induced liposome gels to serve as injectable biomaterials. The physical bonds holding the gel are susceptible to shear and so the gels exhibit shear-thinning behavior, which permits them to be injectable. At the same time, the gels recover rapidly from shear and achieve sufficiently high elastic moduli to ensure localization at the injection site (see Figure 1). We show that cationic solutes such as the anti-cancer drug, doxorubicin (Dox) can be encapsulated in these liposome gels, and our kinetic studies show that the release of solute from the gels can be sustained for over a week. Thus, hmC-connected liposome gels are shown to be a promising class of injectable biomaterials.

Materials and Methods

Materials

All chemicals were purchased from Sigma-Aldrich unless specified otherwise. All lipids were purchased from Avanti Polar Lipids, including egg phosphatidyl choline (egg PC), dipalmitoylphosphatidyl-choline (DPPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000). Doxorubicin hydrochloric acid was obtained from Bedford Laboratories. Doxil[®] was purchased from Ben Venue Laboratories. Doxil[®] liposomes are composed of N-(carbonyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine sodium salt (MPEG-DSPE), 3.19 mg/mL; fully hydrogenated soy phosphatidylcholine (HSPC), 9.58 mg/mL; cholesterol, 3.19 mg/mL; and doxorubicin HCl, 2 mg/mL. Chitosan of molecular weight 200 kDa and 80% degree of deacetylation was used. The hmC used in this work was identical to that used in a previous paper and was obtained by grafting n-dodecyl hydrophobes to the above chitosan at a graft density of 2.5 mol% (relative to the available amines).²⁴

Preparation of liposomes

Small unilamellar vesicles (SUVs) (20 mg/mL) were prepared as follows. First, a lipid film was formed by removing chloroform solvent under nitrogen at room temperature. Residual chloroform was removed by placing the films overnight in a lyophilizer. To this film, a solution of ammonium sulfate (300 mM, pH 7.5) was added,^{25–27} and liposomes were prepared by probe sonication. The liposomes were then eluted through a size exclusion gel chromatography column (Biogel A-0.5m, Bio-Rad Laboratories) preequilibrated with pH 7.4 HEPES buffer. The liposomes were collected and incubated with Dox overnight at room temperature. In this way, Dox-containing liposomes were prepared, and they were in the size range of 100 – 150 nm diameter, as determined by dynamic light scattering. Liposome stability was assayed by determining leakage of Dox in PBS. To measure Dox leakage and concentration, various fractions were analyzed for Dox concentration in the presence of Triton X 100 (TX100) as above. A calibration curve for the Dox concentration was made from fluorescence intensity readings (Excitation 480nm/Emission 580nm) (1–40 μ M, $r^2=0.995$). We used Dox fluorescence intensity of each sample in the presence of Triton X 100 (TX100) as 100% entrapment efficiency.

Liposome characterization

A Malvern Zetasizer Nano ZS instrument was used to measure the hydrodynamic diameter of the liposomes at 25°C. Three measurements were made per sample and the averaged data was reported.

Preparation of hmC-liposome gels

Liposome and polymer mixtures of desired composition were prepared by mixing the corresponding stock solutions based on the previous CTAT/SDBS vesicles+hmC study.²⁴ Gels can form in a broad composition range, either at higher hmC+ lower liposome concentration or at lower hmC+ higher liposome concentration. The liposome and hmC concentrations were varied over a wide range and the optimum compositions have been chosen for each experiment. For example, an optimum composition of Dox-encapsulated DPPC liposomes (Dox-lipo) (lipid conc. 15 mg/mL, Dox conc. 12 μ g/mL) and hmC (10mg/mL) was used for the rheological study. For Doxil+hmC, an optimum composition of Doxil[®] (Dox conc. 780 μ g/ml and lipid conc. 5mg/ml) + hmC (17mg/mL) was chosen for comparison experiment. After that, samples were vortexed and homogenized with handheld homogenizer (Fisher Scientific), followed by centrifugation to remove bubbles.

Rheological studies

Steady and dynamic rheological experiments were performed at 25°C on an AR2000 stress controlled rheometer (TA Instruments). A cone-and-plate geometry of 40 mm diameter with a 2 degree cone angle was used. Dynamic frequency spectra were obtained in the linear viscoelastic regime of the samples, as determined by dynamic stress sweep experiments.

Cryo-TEM

4 μ L of sample were blotted onto glow-discharged holey carbon grids R2/2 (Quantifoil) and vitrified in a controlled environment (21°C, 100% rel. humidity) using a Vitrobot vitrification device (FEI). Images were recorded with a T20 microscope (FEI) at 200 kV on an Eagle CCD camera (FEI) while sample temperature was maintained below -170°C in a Gatan 626 cryoholder.

In vitro release studies

Dox-lipo+hmC liposome gel with free Dox present (DPPC 10mg/mL, Dox 26 μ g/mL, hmC 10mg/mL) and with free Dox removed (DPPC 10mg/mL, Dox 2 μ g/mL, hmC 10mg/mL) were prepared. Samples of 1 mL were placed in the bottom of a 3.5 mL vial and PBS was added on top. The supernatant of the vial kept at $37 \pm 0.1^{\circ}\text{C}$ was repeatedly monitored using a fluorescent microplate reader (SpectraMax M2, Molecular Devices) by measuring doxorubicin fluorescent intensity (Excitation 480nm/Emission 580nm) as described above. The withdrawn solution was recovered to the vial after measurement. Cumulative release % was plotted by converting the fluorescent intensity to the corresponded Dox concentration and calculating the ratio of the Dox concentration to the initial drug concentration. Three measurements were made per sample and the average data and standard deviation of four samples were reported.

Cell viability tests

Cytotoxicity by diffused drug through Corning Transwell filter was evaluated using SK-BR-3 cell lines. Human breast adenocarcinoma cell (SK-BR-3, ATCC HTB-30™) was purchased from the American Type Culture Collection (ATCC, Manassas, VA). 1.2×10^6 cells were seeded per well and 2 mL DMEM media was added to each well. The liposome gels were prepared by mixing hmC (13mg/mL) + Doxil® (Dox conc., 280–400 μ g/mL, Dox to lipid ratio 1:6.4). In this experiment, commercially available Doxil formulation was used for rapid applications and its high Dox concentration, considering that it is not easy to reproduce high Dox encapsulated liposome in the lab. After cells were grown to 70% confluence, 200 μ L of sample (e.g., gel of Doxil® + hmC or a control) was added to the upper chamber above the polycarbonate membrane (0.4 μ m). Additional media (0.8 mL) was also added to the upper chamber. Each day media was replaced with fresh media for 10 days. The cultures were maintained in a humidified incubator at 37°C with 5% CO₂. Cytotoxicity by Dox diffusion from the upper chamber was evaluated by trypsinizing adherent cells and counting cells after trypan blue staining. Three measurements were made per sample and the average data and standard deviation of three samples were reported.

Data Analysis

Data are expressed as mean $\pm$ SD. Cell viability data are expressed as percentage of average values of the corresponding control SK-BR-3 cells. Statistical analysis was performed using ANOVA and the Student's t test was performed for unpaired data between two groups. All tests were two-sided, and a probability value (P) of less than .05 was considered significant.

Results and Discussion

Rheology and structure of liposome gels

Figure 2 shows the results of combining either a Dox solution (780 $\mu\text{g/mL}$) or a solution of Doxil[®] (Dox conc. 780 $\mu\text{g/mL}$ and lipid conc. 5mg/mL) with 17mg/mL hmC. Doxil[®] refers to a commercial doxorubicin-encapsulated liposomal formulation that is widely used in chemotherapeutic treatment of various cancers. A mixture of phospholipids and cholesterol is used to formulate the Doxil[®] liposomes (see details in Experimental Section) and the total concentration of these lipids is 12.8 mg/mL. The mixture of Dox with hmC (Dox+hmC) results in a viscous solution that flows readily in the tilted vial as seen on the photograph in Figure 2a. In contrast, the mixture of Doxil[®] and hmC (Doxil+hmC) shows the properties of an elastic gel that can hold its weight in the tilted vial, as shown on the photograph in Figure 2b. The rheological differences evidenced by these visual observations are quantified by dynamic rheology via measurements of the elastic G' and viscous G'' moduli as functions of frequency. The Dox+hmC mixture shows a viscous response with G'' exceeding G' over the range of frequencies tested and strong dependency of both moduli on frequency. On the other hand, the Doxil+hmC mixture shows a gel-like response: i.e., that at low frequencies, the value of G' exceeds that of G'' and both moduli are nearly independent of frequency. The plateau value of G' as $\omega \rightarrow 0$, is the gel modulus G_0 and its value for this sample is ca. 8.0 Pa.

The injectability of the above Doxil+hmC gel is demonstrated by steady-shear rheology (Figure 3). Here, the viscosity η is plotted as a function of shear rate for the Doxil+hmC gel and the Dox+hmC solution. The Dox+hmC solution shows an initial Newtonian plateau in its viscosity (ca.30 Pa.s) followed by weak shear thinning. In comparison, the Doxil+hmC gel has a much higher viscosity at low shear-rates (~ 70 -fold higher than the above solution), followed by strong shear-thinning. At a shear-rate of around 5 s^{-1} , the viscosity is reduced 100-fold by shear relative to the initial plateau value. The lower viscosity under high shear allows the Doxil+hmC gel to be delivered by injection. To practically test injectability the gels were also successfully pushed through a 30G1/2" syringe needle with little effort.

We also obtained similar results in the case of a sample of Dox-lipo liposomes combined with hmC. When the above liposomal solution is combined with 10mg/mL hmC, we again obtain a weak gel, and the dynamic rheology of this gel is shown in Supporting Information, Figure S1. This gel also shows shear-thinning in steady shear or under large-amplitude oscillatory shear. Figure 4a shows the elastic modulus G' and the viscous modulus G'' of the above gel as a function of oscillatory stress amplitude. The gel remains in the linear viscoelastic regime up to $\sim 56 \text{ Pa}$ (strain 430%). At stresses larger than 56 Pa, G'' becomes greater than G' , indicating disruption of the liposome-hmC network. This again shows that the gel can be delivered by injection.

To be used as an injectable material, it is also important for the Doxil+hmC gel to quickly recover to its initial state after shear is stopped. Figure 4b demonstrates the recovery of the gel following disruptive shear. A pulse of large-amplitude oscillatory shear at an amplitude of 150 Pa was applied for 2 min to disrupt the gel network (middle panel). During this period, the sample shows viscous behavior ($G'' > G'$, with the G' becoming too small to detect accurately). Next, we switched to a low stress amplitude of 1 Pa, which is well within the linear regime of Figure 4a, to study the recovery of the gel. We see from the right panel that the gel-like character ($G' > G''$) is recovered instantaneously and both G' and G'' return to their original values very quickly (within $\sim 100 \text{ s}$). This indicates that the hmC-liposome network is re-established quickly after shear is stopped. The implication is that if a liposomal gel is injected into a body cavity, it will then remain localized at the point of injection due to its gel-like property.

Next, the nanostructure of a Doxil+hmC sample was analyzed by cryo-TEM (Figure 5). The control image of Doxil[®] liposomes alone (Figure 5a) clearly shows many unilamellar liposomes with an elongated shape. The rod-like structure at the centers of these liposomes are crystals of Dox. Similar structures have been seen before by cryo-TEM in Doxil[®] samples.^{28, 29} Figure 5b shows a typical cryo-TEM image of the Doxil+hmC gel. Here again, we see the presence of intact Doxil[®] liposomes in the gel, although the contrast in the image is reduced due to the gel sample being relatively thicker. Previous cryo-TEM images of hmC induced CTAT+SDBS vesicle gels have also shown the presence of intact vesicles.³⁰ The hmC polymer cannot be seen in these images because the chains are not sufficiently thick. However, the fact that intact liposomes are present in the gel leads us to conclude that the gel is formed by the bridging of liposomes by hmC chains, as shown schematically in Figure 1.

Doxorubicin release from liposome gels

Next, we examined the release of Dox from an hmC-induced liposome gel into an external solution of PBS buffer. The Dox-lipo+ hmC gel was selected as a better candidate for in vitro release experiment rather than the counterpart with Doxil, considering that the Dox concentration in the Doxil[®] might vary due to batch-to-batch difference. For these experiments, the gel (volume 1 mL) was placed at the bottom of a 3.5 mL vial and PBS buffer was added on top, as shown by Figure 6. Note from the photograph that the gel is stable and does not get diluted by the added buffer solution. This is because the hmC is insoluble at the pH 7.4 of the buffer (the pH of an hmC sample is less than 6.5 due to the 1% acetic acid used to dissolve the hmC initially). Thus, there is a sharp boundary between the Dox-lipo+hmC gel and the PBS. Of note, the mixture of normal chitosan and liposome does not provide a gel after adding pH 7.4 PBS. The entire vial was placed in a water bath at 37°C, and the drug release was monitored as a function of time.

Dox release from the Dox-lipo+hmC gel (red curve in Figure 6) proceeds slowly and saturates at ~ 50% of the Dox in the sample by about 7000 min. At this point, the detergent Triton X 100 (TX 100) was added to the PBS (supernatant). This molecule is expected to diffuse into the liposome gel and disrupt the liposomes therein. This should cause the Dox encapsulated in the liposomes to leak out, resulting in an increase in Dox release rate into the supernatant. Indeed, we observe precisely this in Figure 6. In turn, it implies that the liposomes are intact in the gel throughout the release experiment before addition of TX 100. Of note, the Dox release from the Dox-lipo+hmC gel is much slower than the Dox release from the Dox+hmC mixture ($p < 0.01$) (data not shown).

In the above case, the Dox-lipo+hmC gel contains Dox both within the interior of the liposomes as well as in the liquid surrounding the liposomes (i.e., “free” Dox). Evidently, it is the free Dox that will be released initially whereas the encapsulated Dox will have to traverse the liposomal bilayer first and then diffuse out through the gel. We also studied the release profile from a Dox-lipo+hmC gel prepared after free Dox separation (in this case, the free Dox was removed by passing the Dox-lipo through a size-exclusion column prior to gelation with hmC). This curve, shown in blue in Figure 6, saturates at ~ 20% of the Dox in the sample, which is much lower than that from the sample for which free Dox was not separated. The lower value is consistent with the negligible amount of free Dox in this sample. Once again, at the 7000 min mark, we introduce TX100 into the buffer, and this causes a spike in the release curve, consistent with disruption of liposomes by TX100 diffusing into the gel. Note that about 80% of the drug in the sample gets released this way, implying that most of the drug did remain in the liposomes. In turn, this shows that liposomal encapsulation of Dox allows for prolonged and sustained release. Release kinetics of Dox from a Doxil+hmC liposome gel was similar to the curves in Figure 6.

The cytotoxic nature of Doxil+hmC gels was evaluated by a Transwell® assay. Initially 1.2×10^6 cells were plated in each well and a sample of interest was added to the upper chamber (see Figure 7a). After 11 days, the cells were counted by trypan blue staining and the graph in Figure 7b shows the percentage of viable cells. Sample 2 (hmC solution alone) shows mild cytotoxicity (killing around 40% of cell) presumably due to the acidity of the solution ($p < 0.01$). Cytotoxicity of hmC has been studied before, and no toxicity was reported by hmC itself.³¹ Note that sample 3 (solution of hmC and Dox) kills more than 80% of the cells ($p < 0.001$); evidently, the Dox diffuses through the polycarbonate membrane and into the lower chamber. Samples 4, 5, 6 are all Doxil+hmC liposome gels and they all show similar cytotoxic effects as sample 3 ($p < 0.001$). In this case, the Dox must be diffusing through the liposomes and through the polycarbonate membrane, eventually killing the underlying cells. It is interesting to compare wells 1–6 in the photograph in Figure 7c, which was taken after 11 days. The Doxil+hmC liposome gels (wells 4, 5, 6) clearly show a red color in their upper chamber, which is due to Dox, even after 11 days (see also Supporting Information). In comparison, the Dox+hmC solution in well 3 shows only a light pink color, indicating that most of the Dox has diffused out into the lower chamber. In other words, the Doxil+hmC gels still contain some of the cytotoxic Dox even after an extended period of time. This shows the utility of the liposome-gel construct in that it can serve as a drug-releasing depot for sustained release.

Compared with the reported thermo-sensitive chitosan gel formulation that forms a gel with chitosan of a specific molecular weight and β -glycerophosphate (GP) amounts,¹⁸ we found that our liposome gel forms in a broad composition range. In the thermo-sensitive gel, the gel forms by β -GP neutralization. There are no active connections between the chitosan matrix and the liposomes, and the liposomes are passively entrapped in the chitosan gel network. On the other hand, in our liposome gel formulation, the liposomes are actively involved in the gel network formation *via* the hydrophobic interaction and act as crosslinking points. Both can be used for sustained drug release and each has its own advantages depending on the applications. In our liposome induced gel, because the liposomes are actively involved in the formation, this gel can be more useful in liposome formulations and increase the liposome concentration.

Conclusions

We have shown that the associating biopolymer hmC forms gels when combined with drug-encapsulated liposomes. Gel formation is mediated by the anchoring of hydrophobes from polymer chains in the bilayers of liposomes. These gels hold their weight in an inverted vial, but when subjected to shear, the physical bonds in the gel network are disrupted, which leads to shear-thinning and hence a low viscosity. When the shear is stopped, the gel network is quickly re-established. Such gels can thus be used as injectable materials for biomedical applications. The gels can form a drug-releasing depot at the site of injection and the drug encapsulated in the liposomes can be released in a slow and sustained manner over more than a week.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Structure of an injectable liposome gel at rest (left) and under shear (right). The hydrophobically modified chitosan (hmC) is shown with its hydrophilic backbone in blue and the grafted hydrophobes in red. When added to doxorubicin (Dox)-containing liposomes, the hmC inserts its hydrophobes into liposomal bilayers and thereby connects the liposomes into a gel, as shown on the left. When shear is applied, the physical bonds holding the network are disrupted, i.e., the hydrophobes disengage from the liposomes, as shown on the right. In turn, the gel is converted into an injectable viscous solution. When shear is removed, the solution rapidly reverts to the gel state.

(a) Dox + hmC **(b) Doxil + hmC** **Figure 2.**

Dynamic rheology data showing that hmC converts a solution of liposomes into a gel. The elastic modulus G' and the viscous modulus G'' are plotted against frequency ω for two cases: (a) A mixture of Dox+hmC shows a viscous response ($G'' > G'$) and the solution flows readily in the tilted vial, as seen in the photograph. (b) A mixture of Doxil+hmC shows a gel-like response ($G' > G''$) and the sample holds its weight in the tilted vial as seen in the photograph.

Figure 3. Steady-shear rheology data (viscosity vs. shear rate) on Doxil+hmcC gel and Dox+hmcC solution. The shear-thinning response of the Doxil+hmcC gel allows the sample to be delivered by injection.

Figure 4.

(a) Data on a Doxil+hmC liposome gel for the elastic modulus G' and the viscous modulus G'' as functions of the oscillatory stress amplitude. (b) Recovery of gel properties after disruptive shear. G' and G'' are shown vs. time for three consecutive shear regimes (left to right): low shear (1 Pa), a pulse of large-amplitude oscillatory shear (150 Pa), and back to low shear (1 Pa). The data show that G' and G'' of the gel recover rapidly to nearly its initial values after cessation of shear.

Figure 5. Cryo-TEM images of (a) commercial Dox-encapsulated liposomes, Doxil[®] (control) and (b) Doxil+hmC liposome gel. The rodlike structures in the liposomes are crystals of Dox. The image in (b) shows that intact liposomes are present in the liposome gels.

Figure 6.

Dox release profile from Dox-lipo+hmC gels. The photograph shows the boundary between the PBS solution (supernatant) and the gel below. Data are shown for the gel with free Dox present (red) and with free Dox removed (i.e., Dox present only in the liposomes) (blue). The lines are to guide the eye. At the points marked by the arrows, Triton X 100 (TX100) is added to the supernatant. This causes a spike in Dox release in both cases. Each point represents the mean value $\pm$ S.D. from four samples.

Figure 7.

Cell viability results. (a) Schematic of the experimental setup. SK-BR-3 cells are plated per well and a given sample is added to the upper chamber. The samples are: 1) DMEM media (control); 2) solution of 13mg/mL hmC; 3) solution of hmC+ Dox (180 μ g/mL); 4) gel of hmC+ Doxil (Dox, 400 μ g/mL); 5) gel of hmC+ Doxil (Dox, 320 μ g/mL); and 6) gel of hmC + Doxil (Dox, 280 μ g/mL). (b) Cell viability results from trypan blue staining after 11 days. The results show that the Doxil+hmC gels are strongly cytotoxic. Each point represents the mean value $\pm$ SD (N=3). (c) Photographs of the upper chamber of each well after 11 days. The wells containing the Doxil+hmC gels still retain a red color showing that Dox is still present in these cases.