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BIOCOMPATIBILITY STUDIES

Original Research

Cytotoxicity and sustained release of modified divinylsulfone from silk based 3D construct

Tuli Dey[1,3] · Banani Kundu[1,4] · Debanjana Deb[2] · Tanmaya Pathak[2] · Subhas C. Kundu[1]

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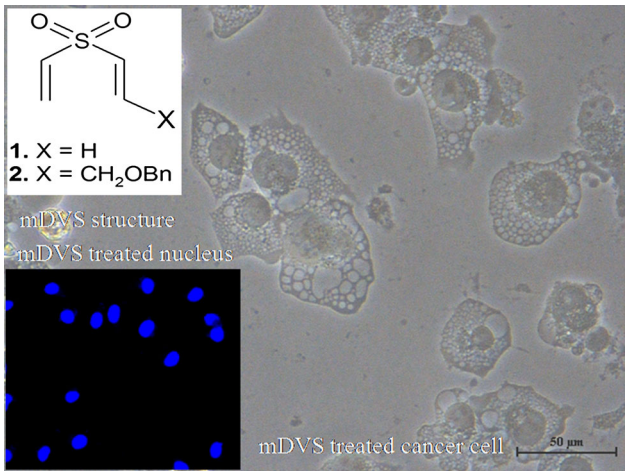
Abstract Monovinylsulfones have been extensively studied for its biological activities but modified divinylsulfones (mDVS**2**) were largely neglected due to the non-availability of appropriate synthetic routes. The present report describes the potential of a unique derivative of divinylsulfone as a remedial molecule. The mDVS**2**, available in reasonably large amount through an easy synthesis route, incites necrosis in invasive and non-invasive breast cancer cells in a time and concentration dependent manner. This molecule is further used to fabricate mDVS**2** embedded silk based 3D scaffolds in order to achieve sustained release. The entrapped molecules retain their activity over time, as 100 % cell death is observed within 7 days. The findings demonstrate the cytotoxic property of mDVS and highlight the importance of under utilized mDVSs as potential therapeutic agents.

Graphical Abstract



1 Introduction

Monovinylsulfones (Fig. 1) are known to inhibit cysteine/serine rich proteases through Micheal addition of the thiol residue on to electron deficient double bonds. These studies have contributed significantly to the identification of new therapeutics [1]. Different biomolecules are functionalized with monovinylsulfone in order to utilize the inhibitory properties of these Michael acceptors [2, 3]. However the potential of divinylsulfone (DVS) or 1,1′-sulfonylbisethene ($H_2C{=}CHSO_2CH{=}CH_2$) 1 (Fig. 2a) as a biologically relevant molecules mostly remains ignored.

In spite of their ability of interacting with wide-ranging nucleophiles in order to form di-substituted or cyclic derivatives, use of the DVS functional group in biology remains limited [4]. This is mostly due to the un-availability of suitable and efficient strategies for the synthesis

✉ Tanmaya Pathak
tpathak@chem.iitkgp.ernet.in

✉ Subhas C. Kundu
kundu@hijli.iitkgp.ernet.in

1 Department of Biotechnology, Indian Institute of Technology Kharagpur, Kharagpur, West Bengal 721302, India

2 Department of Chemistry, Indian Institute of Technology Kharagpur, Kharagpur, West Bengal 721302, India

3 Institute of Bioinformatics and Biotechnology, Savitribai Phule Pune University, Ganeshkhind, Pune 411007, India

4 Institute of Tissue Regeneration Engineering (ITREN), Dankook University, Cheonan 330-714, South Korea

of functionalized divinylsulfones, especially DVS skeleton attached to more complex moieties such as carbohydrates or nucleosides. The number of modified divinylsulfones (mDVSs) subjected to biological studies are even smaller, although a limited number of this class of compounds has been screened as anti-inflammatory and tumor cells growth inhibitory agents [5]. In addition to the limitations of synthetic strategies for accessing mDVSs, it may be argued that erroneous selection of sterically bulky functional groups around the reactive double bonds of an mDVS would lead to misleading conclusions about the potential of these molecules as inhibitory agents [5].

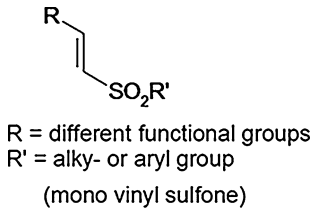


Fig. 1 Structure of monovinylsulfone

A limited number of recent studies are reportedly use divinylsulfone-modified carbohydrates [6] which substantiate the applicability of such compound for bio-medical purposes. Appropriately designed mDVSs, namely divinylsulfone-modified carbohydrates are shown to manifest their potentials as a new class of inhibitors of amoebic parasites [7]. We assume that certain mDVSs would be more efficient than monovinylsulfones because the former class of molecules is capable of alkylating two biological nucleophiles. Our previous success with mDVS-based inhibitors [7] led us to design a new mDVS**2** (Fig. 2a) having two double bonds with different pre-decided reactivity. It is expected that one of the double bonds of mDVS**2** would react faster like any monovinylsulfone to form a covalent bond in the cell. The other less reactive double bond is expected to find another biological nucleophile after the insertion.

Cystein/serine rich proteins such as proteases are the most probable nucleophile target for the divinylsulfone

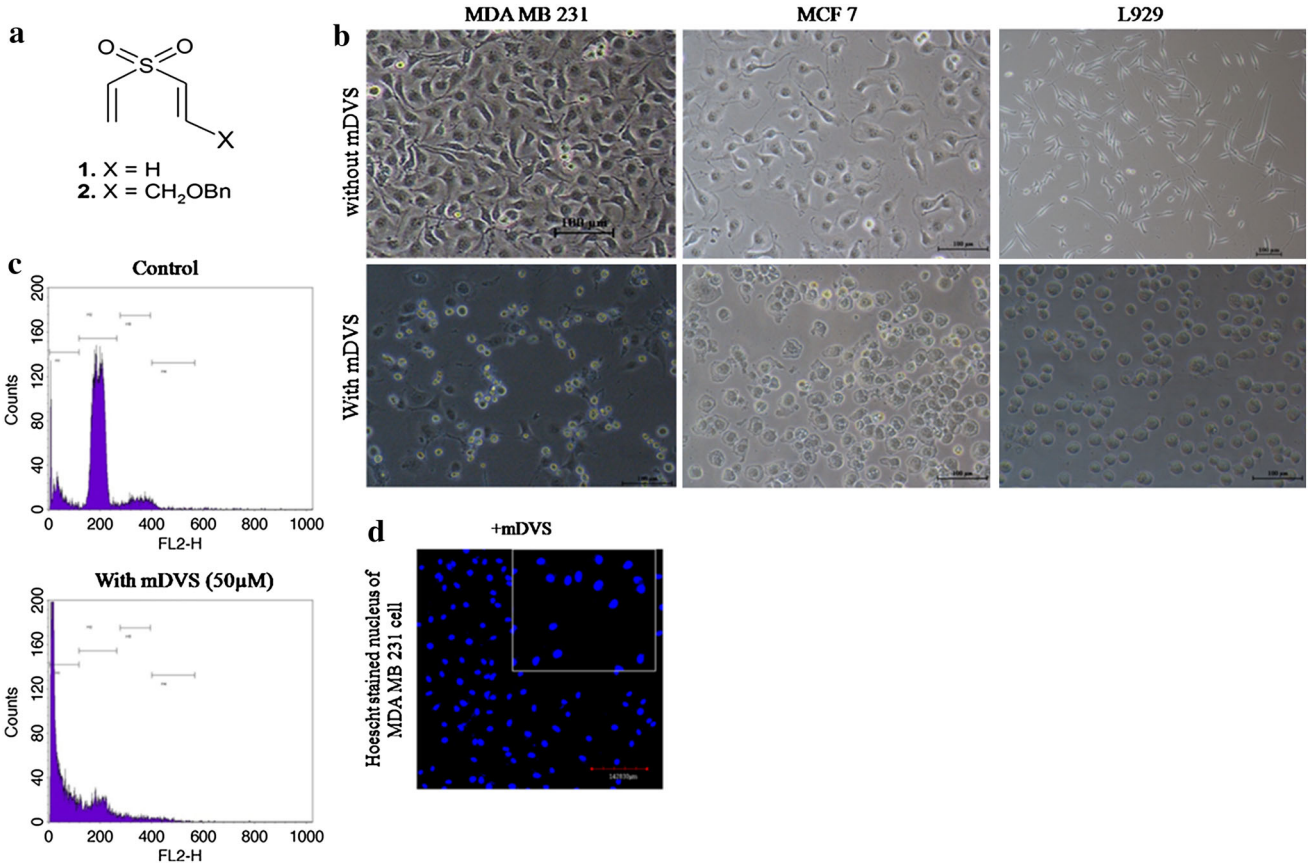


Fig. 2 Cytotoxicity of mDVS**2** against invasive (MDA MB 231), non-invasive breast cancer cell lines (MCF 7) and fibroblast (L929) cell lines. **a** Schematic diagram of mDVS**2** structure, **b** phase contrast image of control and mDVS**2** treated MDA MB 231, MCF7 and L929 cells after 24 h, **c** cell cycle analysis of mDVS**2** (50 µM) treated MDA MB 231 cells. **d** Fluorescence microscopic image of mDVS**2** treated MDA MB 231 cell nuclei

molecules. Cellular proteases represent a vital point for therapeutic intervention through protease inhibitors [8]. Several cysteine protease inhibitors are in different phases of clinical testing [9]. Although specific targets for mDVSs are not known yet, the mitochondrial proteases may respond as potential targets [10]. The conventional protease inhibitors mostly target the cellular and mitochondrial cysteine rich proteases of different classes such as calpains [11] and caspases [12]. Protease inhibitor mediated attack on cellular function invariably affects the mitochondrial permeability transition (MPT), which is followed by necrosis [13]. Nonspecific interaction and degradation are among the common problems of any reactive drug molecules which also include the nucleophiles. The reactive nature of such molecules also renders them unstable in physiological conditions. To circumvent these problems of instability and non-specific interactions, controlled and temporal delivery of such molecules is assumed to be the single answer. Different polymer based delivery system are reportedly use to contain and deliver the drug of interest to the diseased tissue. We and others have used silk protein fibroin based porous scaffolds which has no reported cytotoxic and deleterious effects of its own, for different biomedical purposes including drug delivery [14, 15]. Silk protein based thin film [16], nanoparticle [17] and nanofiber [18] are used successfully by different groups to deliver drug or therapeutically relevant molecules [19] both in vitro and in vivo studies.

In this study, the cytotoxic efficiency of mDVS**2** is evaluated in free form and also embedded within 3D silk fibroin scaffold as a model construct. The following issues are analyzed: (1) in vitro toxicity of the mDVS**2** against breast cancer cell lines; (2) the effect of mDVS**2** encapsulation on silk fibroin based 3D construct (3) release profile of mDVS**2** and (4) in vitro cytotoxicity of mDVS**2** encapsulated 3D fibroin scaffolds.

2 Method and materials

2.1 Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), trypsin–EDTA, penicillin/streptomycin, fetal bovine serum (Gibco, Invitrogen, USA), thiazoyl blue (MTT), aminopterine (Sigma Aldrich, St. Louis. USA), tissue culture grade polystyrene flasks, cell culture plates (Tarsons, India), DAPI (Sigma Aldrich), TRITC-Phalloidin (Invitrogen) and cellulose dialysis membranes (Pierce, USA) were purchased. All other chemicals were of analytical grade and procured from Merck (India) and Sigma Aldrich if not mentioned otherwise.

Fresh mulberry silk cocoons of *Bombyx mori* were obtained from local Farm, West Midnapore District, West Bengal, India.

2.2 Cell culture

MDA MB 231 (epithelial), MCF7 (epithelial) and L929 (fibroblast) cell lines are obtained from National Centre for Cell Science, Pune, India. The cells were maintained in DMEM supplemented with 10 % fetal bovine serum and 1 % penicillin-streptomycin in 37 °C incubator with 5 % CO_2.

2.3 Synthesis of modified divinylsulfone (mDVS2)

Triose derived divinylsulfone was prepared by following the protocol published elsewhere.[6]

2.4 Analysis of mDVS2 mediated cytotoxicity

In vitro cytotoxicity of mDVS**2** against human breast cancer (MDA MB 231 and MCF7) and mouse fibroblast (L929) cell lines were measured through tetrazolium blue mediated cell viability assay. The initial inoculum of 5000 cells/well seeded in 96 well culture plates, cultured for 48 h, followed by 12 h serum starvation. The cells were further treated with mDVS**2** (dissolved in dehydrated ethanol) and left for 24 h. Ethanol and aminopterin (1×) treated cells were used as control. MTT assay was performed as per manufacturer's protocol, by adding 250 µl of tetrazolium blue dye (5 mg/ml stock) to the wells for 4 h. The cytotoxicity was calculated from the optical density (575 nm). Microscopic analysis of cell death was analyzed by seeding approximately 5000 MDA MB 231, MCF and L929 cells/well. After 48 h incubation, 70 % confluent cells were treated with mDVS**2** (IC50 dose) for 12 h and the cells were fixed in 2 % paraformaldehyde (PFA) followed by 15 min incubation with Hoechst 33342 (nucleic acid stain). The cellular morphology of all three cell lines was analyzed by phase contrast microscopy. The nuclei structures of specifically MDA MB 231 cells were analyzed with CLSM (FV1000, Olympus, Japan). MDA MB 231 cell line was used for rest of the process of evaluation for being an aggressive breast cancer cell line in cancer research.

MDA MB 231 cells were seeded in 1×10^5 cell/ml concentration and cultured for 24 h. After 12 h of serum starvation, the cells were treated with 50 µM of mDVS**2**. After 12 h incubation, the cells were trypsinized and fixed with ice cold ethanol. Fixed samples were treated with RNase (10 mg/ml) and PI (5 mg/ml) and subjected to fluorescence activated cell sorter (FACSCalibur, BD Biosciences).

2.5 Fabrication of 3D mDVS2-silk blend scaffold

Aqueous silk protein fibroin solution was prepared following a standard protocol described elsewhere.[14] Briefly the cut pieces of cocoon of mulberry *Bombyx mori* was boiled in 0.02 M Na_2CO_3 for 1 h for degumming (removal of another silk protein sericin). Dissolution of the degummed fiber was carried out using 9.3 M LiBr solution for 4 h at 60 °C. The traces of salts in final solution were removed by extensive dialysis against de-ionized water for 36 h following several changes of water. Fibroin concentration was quantified using colorimetric method (Bradford).

The fabrications of mDVS**2** embedded scaffolds were made by mixing 25, 50 and 100 μM of mDVS**2** with 2 wt% fibroin solution (at physiological pH) under constant stirring at 37 °C for 15 min. Each blended solution (200 μl) was poured into the molds of 96 well tissue culture plates to obtain 3D scaffolds of 5 mm diameter and 2 mm height. Equimolar amount of aminopterine was used as a positive control to fabricate aminopterin entrapped 3D silk scaffolds in the same way. The solution was frozen at −20 °C for 24 h followed by 12 h lyophilization. The pores initiated at this point due to phase separation of silk protein and ice crystals, which further vaporized in vacuum. The porous scaffolds were briefly treated with absolute ethanol for 5 min to induce β-sheet formation and sterilization before cell seeding.

2.6 Characterization of 3D mDVS2-silk blend scaffold

The samples were gold-sputtered and imaging was carried out under scanning electron microscope (JEOL JSM-5800). The pore size measurement was made by selecting 20 random pores of each scaffold types using Image J 1.45 (NIH) software.

The porosity and interconnectivity of conjugated scaffolds were measured by liquid displacement method. The conjugated scaffold was immersed in a known volume of hexane and the porosity (ε) was calculated by

$$\varepsilon(\%) = (V_1 - V_3)/(V_2 - V_3) \times 100$$

where, V_1 is the initial volume of hexane; V_2 is the hexane-impregnated scaffold; V_3 is the residual hexane volume.

Swelling ratio (SR) of the conjugates was examined by incubating the lyophilized scaffolds of known weights (W_0) in phosphate buffer saline (PBS, pH 7.4) at 37 °C and sampled at pre-determined time intervals. The swelled samples were weighed (W_t) again after removing the excess liquid from its surface with the help of tissue paper. The SR was determined as follows: $SR = (W_t - W_0)/W_0$.

Biodegradability of the 3D scaffolds was measured by protease XIV (*Streptomyces griseus*) (2 unit/ml) treatment study. The scaffolds were weighed (E_0) followed by incubation in enzyme solution and 1× PBS (control) solution at 37 °C. The quantitative loss in weight was expressed as percentage (%) of remaining weight (E_t) to initial dry weight.

2.7 Release profiling of mDVS2 from 3D scaffolds

To analyze the release profile mDVS**2** and aminopterin entrapped scaffolds were completely submerged in 1× PBS and incubated at 37 °C for different time points. At fixed interval, solvents were collected and same amount were replenished with fresh PBS. Amount of released mDVS**2** in collected samples was analyzed using UV–Vis spectrophotometer.

2.8 Effect of mDVS2 embedded 3D scaffold on cell viability

Silk-mDVS scaffolds were washed and equilibrated with 1× PBS (pH 7.4) and cell culture medium respectively. Approximately 1×10^5 MDA MB 231 cells were seeded to each equilibrated scaffold.

The cytotoxic efficacy of encapsulated mDVS**2** was examined by calcein-AM/EthD-1 staining (Live/Dead staining kit, Invitrogen) after 5 days of culture using the sequential scanning mode of CLSM (FV 1000, Olympus, Japan).

The morphology of MDA-MB-231 cells seeded on the mDVS**2**-fr scaffolds was assessed by scanning electron microscopy after 7 days of culture.

2.9 Effect of mDVS2 on cellular cytoskeletal system

Silk-mDVS solution was used to prepare 2D thin film using petri plates. MDA 231 cells were seeded on the plates (5000 per cm^2) and incubated under standard conditions for 3 days. The cells were further washed and fixed with 2 % PFA, followed by permeabilization with 0.01 % Triton x100. Cells were stained using TRITC-phalloidin (1:500 dilution) and DAPI (1:500 dilution) for 1 h. Stained cells were further analyzed using CLSM (FV 1000, Olympus, Japan).

2.10 Statistical analysis

All the data represented as mean ± standard deviation ($n = 3$) and all the experiments were repeated thrice. Student's t test is done for 2 sample analysis and ANOVA is performed for multiple sample number to determine the significance level ($p < 0.05$ is considered significant).

3 Result

3.1 Cytotoxicity of modified vinyl sulfone2

In vitro cytotoxicity of mDVs**2** (Fig. 2a) on invasive (MDA MB 231) and non-invasive (MCF 7) human breast cancers and control fibroblast (L929) cells are measured through cell viability assay and phase contrast microscopy. IC_{50} values of mDVs**2** against different cell lines are calculated (Table 1). Morphological changes such as cytoplasmic blebs in both types of cancerous cells are evident from phase contrast microscopy (Fig. 2b). The cell cycle analysis shows that mDVs**2** treated cells significantly increase the population in G_0 fraction in comparison to control ($P < 0.05$) (Fig. 2c). The nuclei of mDVs**2** treated cells show no significant structural difference in comparison to control (Fig. 2d).

3.2 Characterization of mDVS2 embedded 3D scaffold

Morphology of 3D silk scaffolds fabricated with mDVS 2 is analyzed with scanning electron microscopy. The composite scaffolds reveal almost homogenous distribution of pores (Fig. 3a) and show no distinguishable difference in structures in comparison to virgin (pure) silk scaffolds. Quantitative analysis of increased pore size is observed in scaffolds with higher concentration of mDVS**2**. Interestingly porosity or interconnectivity of mDVS**2** entrapped scaffolds indicates that the higher mDVS**2** concentration may induce increased porosity in comparison to control scaffolds (Fig. 3b). Swelling study of mDVS**2** entrapped scaffolds shows rapid initial uptake of the solvents which achieve a saturation plateau within 48 h (Fig. 3c). Pure silk fibroin scaffolds of *B. mori* reveal highest solvent uptake. The composite scaffolds exhibit no significant difference in swelling ratios within themselves. To characterize the fluid uptake nature, the swollen scaffolds are lyophilized. The lyophilization does not show any significant shrinkage in terms of the height or diameter of the composite scaffolds.

Protease mediated biodegradation of composite scaffolds established that the addition of mDVS**2** does not alter the protein structure and protease mediated degradation rate (Fig. 3d).

Table 1 IC_{50} values of mDVS 2 against cancerous (MDA MB 231, MCF7) and Fibroblast (L929) cell lines

MDA MB 231	MCF 7	L929
16 μM	20 μM	48 μM

3.3 In vitro release profile of mDVS2 from 3D scaffold

Release profile analysis shows slow initial release over first 4 days. The elevated release after sixth day is probably through simple diffusion process (Fig. 4) as the structural integrity of the constructs remains unaltered. The release profile of mDVS**2** molecules from the scaffold increase over time after 6 days. The release of control drug molecule, aminopterin from the scaffold remains unchanged over the time. The detectable amount of mDVS**2** molecules over 24 days indicates sustained in vitro release.

3.4 Effect of mDVS2 embedded 3D scaffold on cell viability and cytoskeletal distribution

Modified divinylsulfone embedded scaffold exhibit no significant change in scaffold structure, however the cell proliferation analysis of mDVS**2** and aminopterin entrapped scaffold show significant cell death within the third day of seeding. Approximately 100 % cells are dead on 100 μM scaffolds within 7 days (Fig. 5a). The cell viability study through Live/Dead staining on 3D scaffold over time reveals that mDVS**2** containing scaffolds show lowered (25 μM) or no cell proliferation (100 μM) in comparison to control samples (0 μM). Altered cell morphology in drug entrapped 3D scaffolds is evident which signifies the presence of dying and/or dead cells (Fig. 5b). Distribution of actin in control and mDVS exposed cells show distinct altercation. As observed in Fig. 6 the actin shows aggregated distribution in 25 and 100 μM mDVS in comparison with control actin. The actin cytoskeleton exhibit the cellular shock in terms of actin degradation and loss of cellular contact.

4 Discussion

Monovinylsulfones (Fig. 1) have been extensively studied for their therapeutic properties as cysteine rich protease inhibitors [1]. Due to the limitations of synthetic strategy, modified DVSs remains as an under utilized class of molecules in biological study. Additionally the presence of bulky functional groups around the reactive double bond may hinder their anticipated effect in the previous report [5]. However the evaluation of divinylsulfone-modified carbohydrates shows the potential of this type of compounds as therapeutic agent against gastro-intestinal parasites [7]. It is expected that DVS or mDVS may perform better due to the availability of two reactive groups (Fig. 2a).

The probable pathway of DVS mediated cytotoxicity is not known yet, though cysteine rich proteases belonging to

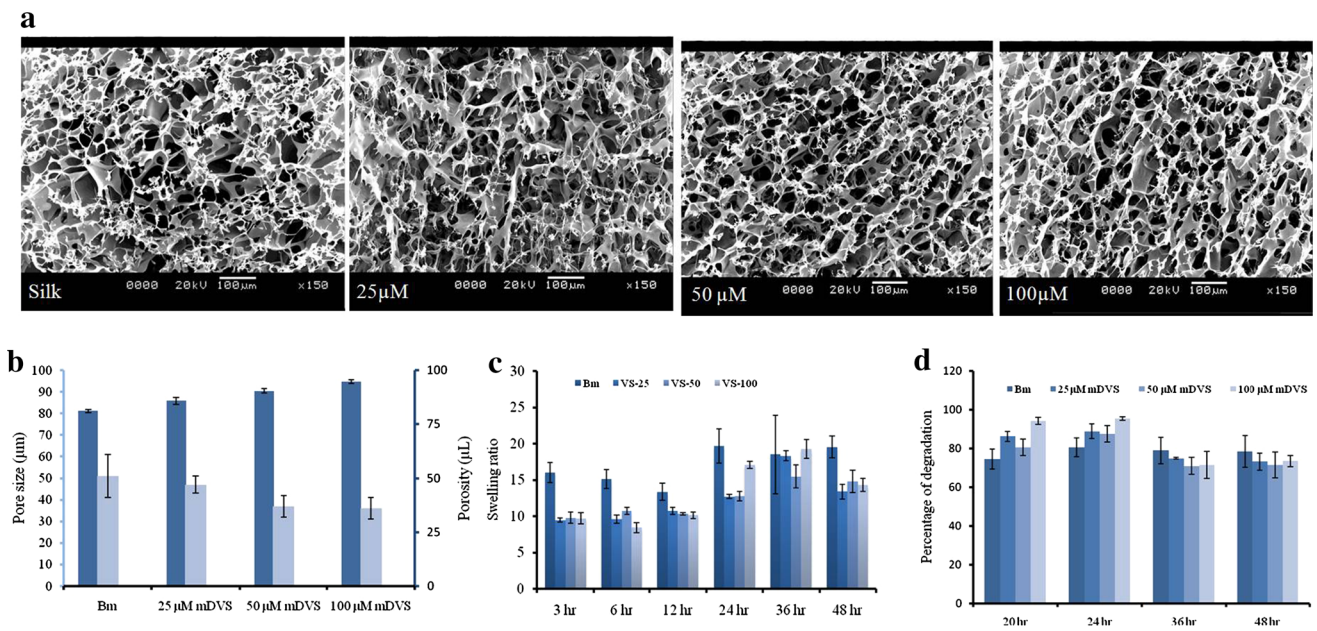


Fig. 3 Morphological and structural characterization of mDVS**2** embedded scaffolds. **a** Scanning electron microscopy of control and drug embedded scaffolds. Scaffold of pure mulberry silk, scaffold of 25 μM mDVS**2**, scaffold of 50 μM mDVS**2**, scaffold of 100 μM mDVS**2**. **b** Analysis of pore size and porosity in control and experimental scaffolds (n = 3). **c** Swelling study of drug entrapped scaffolds (n = 3). **d** Analysis of biodegradability of the composite scaffolds in comparison with control (n = 3)

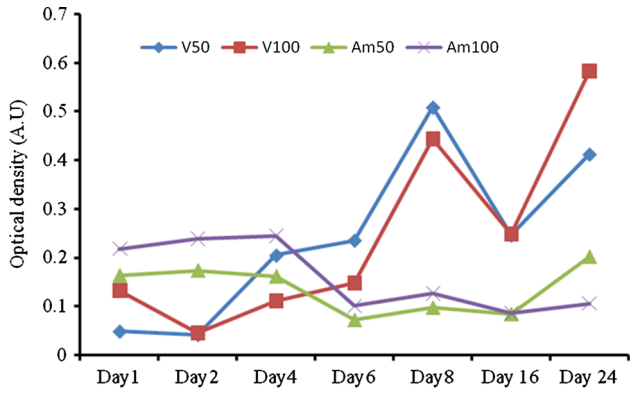


Fig. 4 Time dependent release profiling of mDVS**2** from scaffolds

cytosolic or mitochondrial origin may be categorized as probable target group [10]. Different class of cysteine rich proteases play significant role in cellular function as well as in cellular pathology [11, 12]. Cysteine protease mediated cytotoxicity mostly initiates MPT through cytoplasmic blebbing and mitochondrial swelling. This leads to ATP depletion and cell death [13]. The mDVS**2** treated breast cancer cells (both MDA MB 231 and MCF 7) exhibit initiation of cell death within 12 h of incubation under standard cell culture conditions through distinct cellular disintegration and formation of cytoplasmic blebs (Fig. 2b). Fluorescence activated cell analysis shows abundant cell debri in G_0 fraction (Fig. 2c) but no sign of fragmented nuclei was observed (Fig. 2d). Interestingly cells growing on mDVS releasing surface shows ill formed actin cytoskeleton and reduced cell size. All these observations indicate the necrotic nature of mDVS**2** mediated cytotoxicity.

Additionally silk based 3D scaffolds are used to control the in vitro release profile of mDVS**2**. Interactions between silk protein and mDVS**2** do not occur here as protein-DVS coupling require higher temperature, altered pH and long incubation [20]. Encapsulation of mDVS**2** into silk matrices does not alter the morphology and physical characteristics of the matrices (Fig. 3) but significantly alter the biocompatibility of the scaffold (Fig. 5). The release of mDVS**2** is being controlled primarily through diffusion and subsequent degradation of the matrix (Fig. 4). The diffused amount of mDVS**2** is enough to disrupt the actin cytoskeletal structure within 3 days (in 2D) and eradicate them within 7 days (in 3D). Distressed cell morphology is evident on drug embedded scaffolds which support the cytotoxic nature of the embedded mDVS**2** (Fig. 6). Though the exact pathway of mDVS**2** mediated cell death is not clear, a probable route to initiate programmed necrosis is assumed which need further in depth analysis.

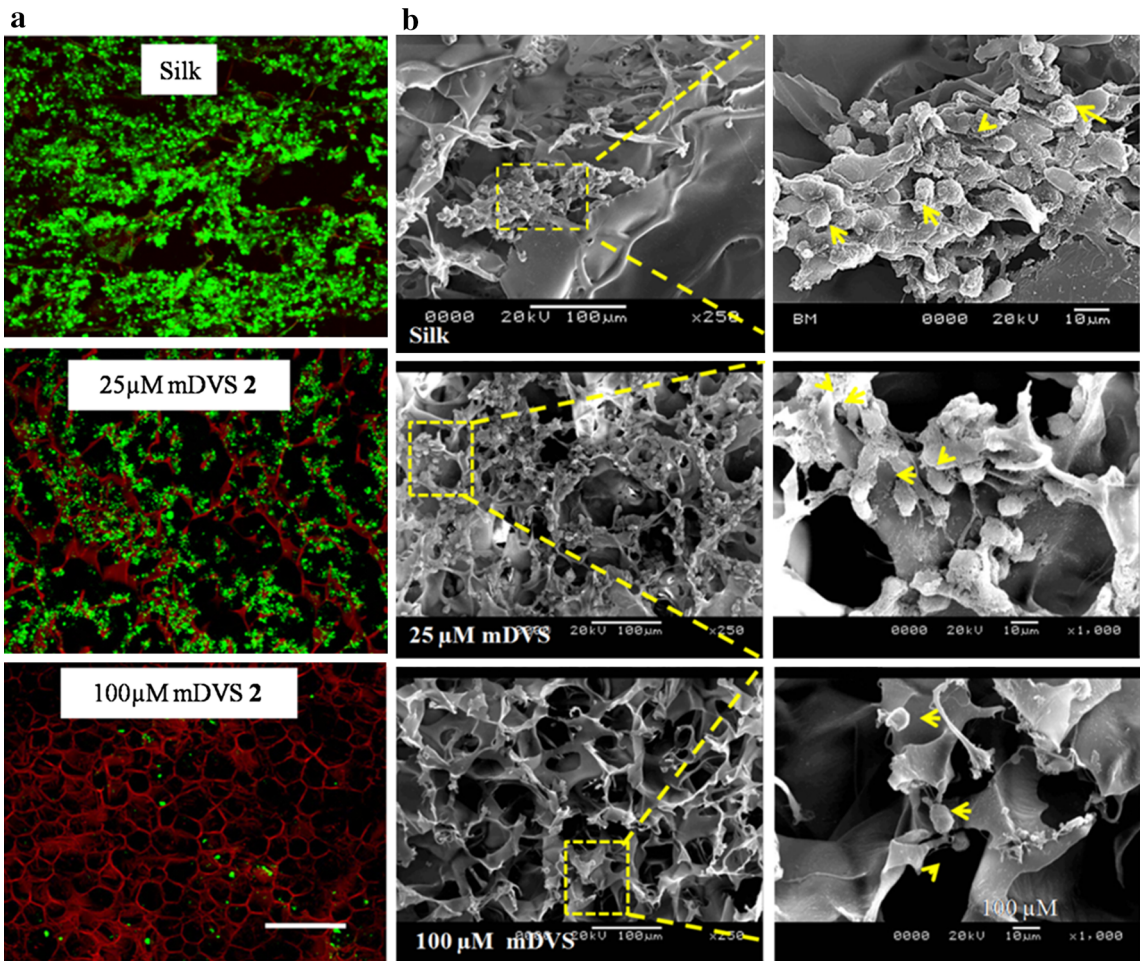


Fig. 5 Analysis of cell proliferation/viability and morphology of MDA MB 231 cells on mDVS**2** embedded scaffolds. **a** Live/dead staining of MDA MB 231 cell seeded scaffold after 7 day of cell seeding. *Scale bar* is 250 μM, **b** Scanning electron microscopic image of MDA MB 231 cell morphology in drug entrapped 3D scaffolds on 7th day. The *yellow arrows* indicate single cell (Color figure online)

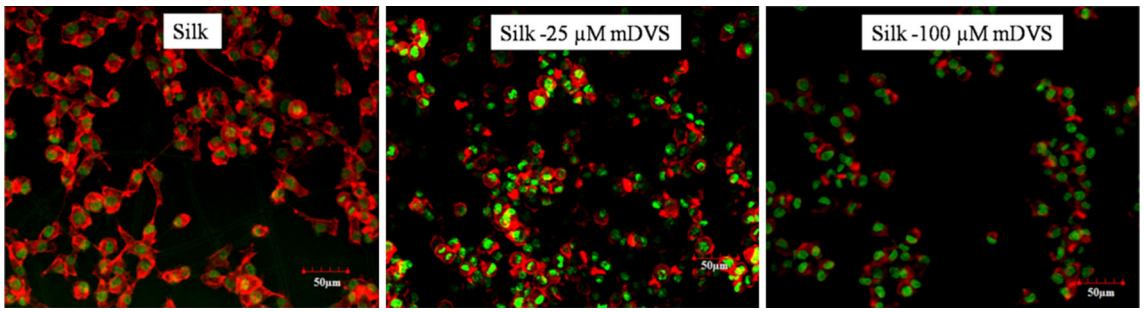


Fig. 6 Cytoskeletal distribution of MDA 231 cells on silk-mDVS coated surface. Cells were seeded on silk-mDVS coated petri plates and incubated for 3 days before fixing them with PFA. Cells were counter stained with TRITC-phalloidin (actin-*red*) and DAPI (nucleus-pseudocolor *green*). Images were obtained using Olympus 1000FV confocal microscopy using 543 and 405 nm. The *scale bar* is 50 μM

5 Conclusions

This study reports the potential of an efficient Michael acceptor mDVS**2** as a therapeutic molecule, which activates necrosis in breast cancer cells. Three dimensional silk scaffolds embedded with mDVS**2** are fabricated to ensure its sustained release, which supports the concept of using bio-active molecule encapsulated scaffolds with tunable biodegradability as a therapeutic construct.

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Compliance with ethical standards

Author Contributions The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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