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A cisplatin slow-release hydrogel drug delivery system based on a formulation of the macrocycle cucurbit[7]uril, gelatin and polyvinyl alcohol

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Abstract

The anticancer drug cisplatin was encapsulated within the cucurbit[7]uril macrocycle to form the host-guest complex: cisplatin@CB[7]. This was then incorporated into gelatin and 0-4% w/v polyvinyl alcohol (PVA)-based hydrogels as slow release drug delivery vehicles. The hydrogels demonstrated predicable swelling and disintegration dependent on the PVA concentration. The hydrogel with the highest PVA content was slower to swell and release drug compared with lower concentrations of PVA. The effect of the hydrogel PVA concentration on in vitro cytotoxicity was examined using A2780/CP70 ovarian cancer cells. Over the 24 h drug exposure time used, hydrogels containing 4% PVA showed a 20% decrease in viable cells compared to the control, whereas hydrogels containing 0% and 2% PVA induced an 80% and 45% inhibition of cell growth, respectively. There was no measurable difference in the in vitro cytotoxicity of free cisplatin and cisplatin@CB[7] containing hydrogels. Finally, the in vivo effectiveness of a 2%-PVA hydrogel implanted under the skin of nude mice bearing A2780/CP70 xenografts showed that low dose hydrogels containing cisplatin@CB[7] (30 µg equivalent of drug) was just as effective as an intraperitoneal high dose administration of free cisplatin (150 µg) at inhibiting tumour growth.

1. Introduction

The major problem associated with the use of platinum-based drugs in the clinic is their severe side effects, which include: neurotoxicity, ototoxicity, myelosuppression, nausea and vomiting [1]. These side effects are so severe that they limit the dose that patients are able to tolerate, and as such, a sub-lethal dose may be delivered to the tumour. Subsequently, a sub-lethal dose of drug administered to the tumour gives rise to drug resistance [2], which is a particular problem in the treatment of ovarian cancer [3]. Therefore, instead of the continued development of new platinum drugs, which might result in drugs that are more cytotoxic but display the same or more severe level of toxicity (i.e. BBR3464) [4], it is important to also develop new delivery systems which can reduce the severity of the drugs' side-effects [5, 6].

There are a number of ways in which the side effects of platinum drugs can be reduced. The first method is through better targeting of the drugs to tumours, thereby leaving healthy tissue unaffected [7]. A number of delivery systems, that target the tumour either passively or actively have been examined, such as: liposomes and micelles [8, 9], polymers [10], nanoparticles [11-13], nanotubes [14] and dendrimers [15], as well as by the use of various targeting molecules, such as: folate and estrogen [16, 17], aptamers [18], antibodies [19], magnetic fields[20] and DNA sequence selective agents [21, 22].

The second method by which the side effects of platinum drugs can be reduced is to change their pharmacokinetics (where and how a drug is transported and excreted in the body). The two biggest problems with platinum drug pharmacokinetics are their short blood serum half-lifes (maximum concentration of cisplatin is achieved in less than 10 min before its serum

concentration drops significantly) and the extent of drug-protein binding (up to 90% of cisplatin is protein bound in the blood stream).

Previously it has been shown that the encapsulation of platinum drugs within the cucurbit[*n*]uril family of macrocycles (Fig. 1)[23] provides a number of benefits, including: a slower rate of reaction with sulfur containing peptides (i.e. glutathione) [24-26], physical stability [27], decreased side-effects [28], increased in vitro and in vivo effectiveness [28] and an ability to overcome cisplatin acquired resistance [29].

Unfortunately, cucurbiturils have little effect on the residence time of cisplatin in blood serum and as the spike in platinum concentration is thought to contribute to the severity of their side effects, as such, a slow release delivery system which sustains the concentration of platinum in the blood stream is therefore needed.

Hydrogels are semi-solid forms of hydrophilic cross-linked polymers that have shown application in localised and slow release drug delivery systems [30, 31], They act as delivery vehicles by trapping small drug molecules within the spaces between the polymer cross links. When placed inside the body, contact with water swells the hydrogels, which increases the gaps between the polymer crosslinks and allows the drugs to diffuse into the blood stream.

Hydrogels can be made from various hydrophilic polymers, including: polyvinyl alcohol, poly(lactide-coglycolide), poly(hydroxyethyl methacrylate) and natural polymers such as chitosan, gelatin and alginate. Polyvinyl alcohol (PVA) and gelatin are attractive polymers for the synthesis of hydrogels as they are already approved excipients for use in dosage

formulation, are considered non-toxic and non-irritant, are stable in solution and the solid state, and are commercially available [32].

In this paper we sought to combine the slow release benefit of hydrogels with the ability of cucurbit[7]uril encapsulated cisplatin (cisplatin@CB[7]) to overcome acquired cisplatin resistance [29] as a two-fold drug delivery system. Here we report the synthesis and effectiveness of PVA and gelatin-based hydrogel slow release systems for cisplatin@CB[7].

2. Materials and Methods

2.1. Materials

Cisplatin, PVA and gelatin were bought from Sigma-Aldrich. Cucurbit[7]uril was bought from Dr Anthony Day, University of New South Wales, Australia. The cisplatin resistant ovarian cell line A2780/CP70 was derived in house at the Beatson Cancer Institute, Glasgow from an A2780 line obtained from Dr R.F. Ozols (Fox Chase Cancer Centre, Philadelphia, PA).

2.2. Hydrogel synthesis

Polyvinyl alcohol (0, 2 and 4% w/v) was dissolved in 10 mL of water with stirring at 130 °C. When fully dissolved, 960 mg of gelatin was added and the mixture was allowed to stir for a further 2 h at 90 °C. The mixture was poured into molded plastic tablet strips and then cooled to room temperature, at which time the formulation turned semi-solid. Samples were stored at 0 °C until needed. To prepare cisplatin and cisplatin@CB[7] hydrogels, the PVA and gelatin mixtures were dissolved in water containing the desired concentration of drug.

2.3. In vitro hydrogel swelling

Hydrogel swelling was monitored over a 7 day period. Hydrogels of a homogenous volume containing 0, 2 and 4% PVA were placed in 200 mL phosphate buffered saline (PBS) at room temperature. The extent of swelling was monitored by recording the weight of the hydrogels at specific intervals.

2.4. Hydrogel surface imaging

The hydrogels were placed on a glass strip and examined under a DXR Raman microscope at 100 times magnification. The pore sizes were analysed and measured using the µView computer program.

2.5. In vitro drug release

Hydrogels of 0, 2 and 4% PVA containing 3 mM cisplatin were incubated in 200 mL of PBS at room temperature. At intervals, 5 mL of solution was extracted and its platinum content determined by inductively coupled plasma mass spectrometry (ICP-MS). An Agilent 7700X instrument, with a micromist nebuliser and an octapole collision cell, was calibrated using solutions prepared from a Spex CertPrep platinum standard at concentrations ranging from 0 – 1000 ppb, containing 2% nitric acid. The platinum drug concentration was determined using the ^{195}Pt isotope. Instrument operating conditions used were 1,550 W RF forward power, 0.85 L min^{-1} plasma carrier gas flow, 0.2 L min^{-1} makeup gas flow, 4.6 mL min^{-1} helium gas flow in the collision cell and 0.1 rps for the nebulizer pump. Sample depth was 8 mm, sample period was 0.31 s and integration time was 0.1 s.

2.6. *Effect of hydrogel on in vitro cytotoxicity*

Cisplatin resistant cells (A2780/CP70) were seeded into 24 well plates at a density of 900 cells per well and allowed to attach and grow for 48 h before drug treatment. Cells were then exposed to either 0, 2 and 4% PVA hydrogels containing either 1 mM cisplatin or 1 mM cisplatin@CB[7] for 24 h at 37°C under a 5% CO_2 atmosphere. The medium containing drug was then removed and fresh medium was supplied to the cells and re-incubated for a further 72 h. On the final day, dead cells were washed away with cold PBS, and the remaining cells fixed with methanol and stained with crystal violet blue. Cells were then dissolved in DMSO and the fluorescence reading of each plate was recorded at 590 nm.

2.7. *In vivo effectiveness*

Monolayer cell cultures were harvested with trypsin–EDTA and resuspended in PBS. For the A2780/CP70 xenografts about 10^7 cells were injected subcutaneously into the right flank of athymic nude mice (CD1 *nu*/*nu* mice from Charles River). After 7 to 10 days when the mean tumour diameter was at ≥ 0.5 cm, animals were randomized in groups of 6. Mice were then treated on day 0 with either a single intraperitoneal dose of saline, cisplatin (150 μg) or implanted with a single 2% PVA hydrogel containing either free cisplatin (30 μg) or cisplatin@CB[7] (30 μg); each 30 μg hydrogel equates to approximately 1.2 mg/kg total delivered dose per animal. To implant the hydrogels a small incision was made in the skin of the mice near the tumour, the hydrogels were placed under the skin, which was then resealed with a clamp. Mice were weighed daily to monitor for toxic side effects and used as a general measure of systemic tolerability in the mice; a drop of 10% is used as the maximum tolerated

dose with a weight drops less than 10% considered tolerable [15].Tumour volumes on each day were estimated by calliper measurements assuming spherical geometry (volume = $d^3 \times \pi/6$).

3. Results and discussion

3.1. Hydrogel synthesis

Hydrogels were synthesized by dissolving PVA in hot water containing drug. Once fully dissolved, gelatin was then added to the mixture. The resultant solutions were then poured into plastic tablet strips and subsequent cooling to room temperature resulted in semi-solid, transparent hydrogels (Fig. 2). Each hydrogel had either dimensions of 4 x 7 mm which were used for swelling, disintegration, drug release and in vitro studies or 5 x 10 mm which were used for the in vivo studies. Three types of hydrogels all containing gelatin but with varying concentrations of PVA: 0, 2 and 4% w/v, were synthesised. The hydrogels were stored in the freezer until required and then thawed for 2 h before use.

3.2. Hydrogel swelling and drug release

Hydrogel swelling was examined in PBS by measuring the mass of each individual hydrogel over a seven day period. The results indicate two sequential events taking place. Initially the hydrogels swell with water growing up to 250% of their original size. At their maximum size a tipping point is reached and the absorption of more water results in the gradual disintegration of the hydrogel until it loses all of its physical structure (Fig. 3).

Even within the first 3 h differences in the swelling rates between the three hydrogels can be observed. The gel with no PVA has a swelling peak time of 24 h after which it disintegrates over the next three days. The gel with 2% PVA swells for twice as long (48 h) but disintegrates over the same length of time (three days). The gel which contains 4% PVA displays the longest peak swelling time of 3.5 days and has not fully disintegrated even by day six.

These results demonstrate a clear trend between the PVA content and the rate of hydrogel swelling and disintegration. It was therefore of interest to determine if the rate of drug release from the hydrogel correlated with the rate of swelling.

Hydrogels with either 0, 2 or 4% w/v PVA and with an effective drug concentration of 3 mM were incubated in PBS at room temperature at intervals for up to 24 h. Drug release is observed within 10 min and continues over the next 24 h (Fig. 4). Little difference is observed in the amount of drug that is released between the 0 and 2% PVA hydrogels over the 24 h period. The 4% PVA hydrogel follows the same trend as the 0% and 2% hydrogel in the 1st h, in which there is a similar level of drug release; however, compared to the 0% and 2% PVA hydrogels which show a continued and gradual increase of cisplatin release, the 4% PVA hydrogel reaches its peak drug release at 1 h after which drug release in the solution does not increase. This could be a result of the much slower swelling and disintegration of the 4% PVA hydrogel compared with the other hydrogels.

3.4. Hydrogel surface features

The swelling, disintegration and drug release results may possibly also be explained in part by the varying porosity of the hydrogels with different PVA concentrations. From optical

microscope images of intact hydrogels, significant surface differences are observed for each hydrogel with the average surface indentation size decreasing with increasing PVA content (Fig. 5). The surface of the 0% PVA hydrogels is characterised by a small number of very large indentations with diameters between 38 - 45 µm. In contrast, the 2% PVA hydrogels have many more indentations but they are considerably smaller; 4 - 11 µm in diameter. The 4% PVA hydrogels show almost a perfectly smooth surface with few indentations which average less than 1 µm in size.

3.5. In vitro cytotoxicity

The effect of the hydrogels on the cytotoxicity of free cisplatin and cisplatin@CB[7] was determined using in vitro growth inhibition assays with A2780/CP70 cisplatin-resistant ovarian carcinoma cells. In testing the in vitro cytotoxicity of the hydrogels 24 well plates were used to incubate the cancer cells and administer the gels. For each plate, individual hydrogels were placed inside a permeable insert, which were then fully submerged into separate wells. Each well contained the cancer cells and media (Fig. 6). As media is absorbed by the hydrogels within the insert, they slowly swell and disintegrate, releasing the drug to the outer well where it could then be taken up by the cancer cells.

Cytotoxicity is expressed as the cell viability as a percentage of growth inhibition compared with untreated cells (Fig. 7). From the results it is clear there is a direct correlation between the amount of PVA in the hydrogels and their ability to inhibit growth. Hydrogels containing 0% PVA were the quickest to swell and disintegrate, and the fastest to release the encapsulated drug. They induced the highest cytotoxicity by inhibiting cell growth by 81%. In comparison,

hydrogels containing 2% PVA showed a moderate inhibition of cell growth by 45%, whereas hydrogels containing 4% PVA released the smallest amount of drug and induced an inhibition of cell growth by 20%. A large variation of cytotoxicity is seen in hydrogels of different PVA concentrations; the higher the PVA concentration, the lower the cytotoxicity, which is potentially from the slower rate of release of the drug. The results also show that there appears to be no significant difference between the cytotoxicity of hydrogels which contain either free cisplatin or cisplatin@CB[7], at each PVA concentration. Previously, we found in other in vitro experiments no difference in cytotoxicity between free cisplatin and cisplatin@CB[7] when tested in vitro and a difference was only observed when the two were compared in vivo. The in vitro results here for the hydrogels containing either free cisplatin or cisplatin@CB[7] are therefore consistent with other in vitro results reported [29].

The reduction in cytotoxicity as the amount of PVA increases is consistent with a slower rate of release of the drug from the gel. In the short incubation time of this experiment (24 h) slower release of the drug means there is an insufficient amount of drug and insufficient amount of time for the drug to be taken up into the cells and effect apoptosis through DNA binding. The results do show that regardless of the PVA concentration used, drug is released at a rate to have a measurable effect on cell growth compared with untreated cells. In addition, when the slow swelling, disintegration and drug release rates are taken with the cytotoxicity results, the 2% PVA hydrogels are most suitable as they have moderate in vitro cytotoxicity without swelling, disintegrating and releasing drug too fast or too slow.

3.6. In vivo effectiveness

To examine the in vivo effectiveness of the hydrogels, nude mice bearing A2780/CP70 xenografts were treated on day 0 with a single intraperitoneal injection of either saline or free cisplatin (150 µg: 6 mg/kg) or implanted with a single hydrogel containing 2% PVA and either free cisplatin (30 µg) or cisplatin@CB[7] (equivalent to 30 µg of cisplatin) (Fig. 8). With an average body weight of 25 grams per animal each hydrogel implant gives a total drug dose of ~1.2 mg/kg, which is well below the maximum tolerated dose of cisplatin in this bred of animal [33]. As such, the hydrogels were well tolerated by the mice with no significant change in body weight in the first two days after implantation.

Because of the slow release nature of the hydrogels, their effect on tumour growth delay was monitored over a period of two weeks rather than one week as was previously undertaken in earlier work compared to the effectiveness of cisplatin@CB[7] and free cisplatin by intraperitoneal administration [29]. In this current work, and because the xenograft is highly cisplatin resistant, a high intraperitoneal dose of cisplatin (150 µg per animal) was needed to delay tumour growth significantly (tumour grew to only 42% size of the control tumours over the same time period). When free cisplatin is administered via a 2% PVA hydrogel and at a dose of 30 µg per animal, there is no measurable delay in tumour growth compared with the saline control. In contrast, at the same 30 µg equivalent dose of cisplatin, the cisplatin@CB[7] hydrogel has the same effectiveness as high dose free cisplatin. This result clearly demonstrates the benefit of slow release from the hydrogel. At 1/5 the dose of the non-hydrogel drugs, it is just as effective, but importantly the side-effects are likely to be less severe. The implications of

this could lead to better quality of life for patients during treatment or potentially greater effectiveness of the drugs when administered at high doses.

4. Conclusions

Hydrogels consisting of gelatin and varying concentrations of polyvinyl alcohol were synthesised as a slow release drug delivery system for cucurbit[7]uril encapsulated cisplatin. The hydrogel's rate of swelling and degradation is related to the PVA content with the hydrogel containing the highest PVA content (4% w/v) displaying the slowest swelling, disintegration and release properties. Drug loaded hydrogels also displayed cytotoxicity toward human ovarian cisplatin-resistant cancer cells A2780/CP70 dependent on their PVA content. The most cytotoxic are the hydrogels with low PVA content (0 and 2%) because they release the drug at a rate sufficient to induce cell growth inhibition in the short time of the in vitro assay. The in vivo results demonstrate the success of the hydrogel; low dose formulation of cisplatin@CB[7] in the 2% PVA hydrogel (30 µg per animal) was able to overcome resistance and was just as effective at inhibiting tumour growth as high intraperitoneal dose cisplatin (150 µg per animal). Overall the results suggest an ability of the hydrogel to treat cancers with much lower doses than conventional treatment, thereby greatly reducing the severity of the side-effects experienced by patients and improving their quality of life during treatment.

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Figure captions

Fig. 1. The chemical structures of (a) the anticancer drug cisplatin, (b) the subunit of the polymer polyvinyl alcohol, where n = 500-5000 and (c) the cucurbit[*n*]uril family of macrocycles, where *n* = 5-8, 10 or 14.

Fig. 2. An example of a PVA and gelatin based hydrogel (left panel) and the plastic tablet strip used in molding the hydrogels (right panel).

Fig. 3. PVA hydrogels in PBS, showing the effect of PVA content: 0% (red, ■), 2% (green, ◆) and 4% (blue, ●) on the rate of swelling and disintegration.

Fig. 4. Drug release rates from the hydrogels containing either 0% (red, ■), 2% (green, ◆) and 4% (blue, ●) PVA incubated in PBS over a period of 24 h demonstrating similar drug release for the two lowest PVA hydrogel concentrations and a significantly lower drug release for the 4% hydrogel.

Fig. 5. Optical microscope images of 0% PVA (top panel), 2% PVA (middle panel) and 4% PVA (bottom panel) demonstrating the differences in surface features between the hydrogels.

Fig. 6. Examples of the 24 well plates used to examine the in vitro cytotoxicity of the hydrogels showing the inserts with inlet channels containing a hydrogel (left panel) and the outer wells with just media in the first column and with fully submerged hydrogel containing inserts in the second, third and fourth columns of the plate (right panel).

Fig. 7. The relative cell growth inhibition of ovarian A2780/CP70 cells by the hydrogels compared to untreated cells, showing a correlation between the amount of PVA and the level of cytotoxicity. The green (left) columns are for free cisplatin containing hydrogels and the blue (right) columns are for cisplatin@CB[7] containing hydrogels.

Fig. 8. Drug in vivo cytotoxicity against the human ovarian A2780/CP70 cisplatin resistant tumour xenograft, showing: intraperitoneal free cisplatin (30 µg) hydrogel (purple, ●), saline control (blue, ◆), cisplatin@CB[7] in 2% PVA hydrogel (30 µg, green, ▲) and free cisplatin (150 µg, red, ■).

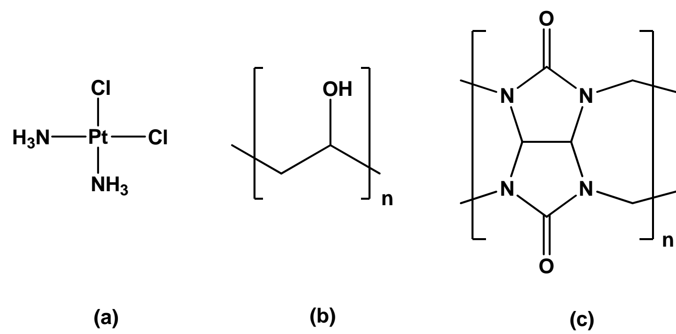

Figure 1

Figure 2

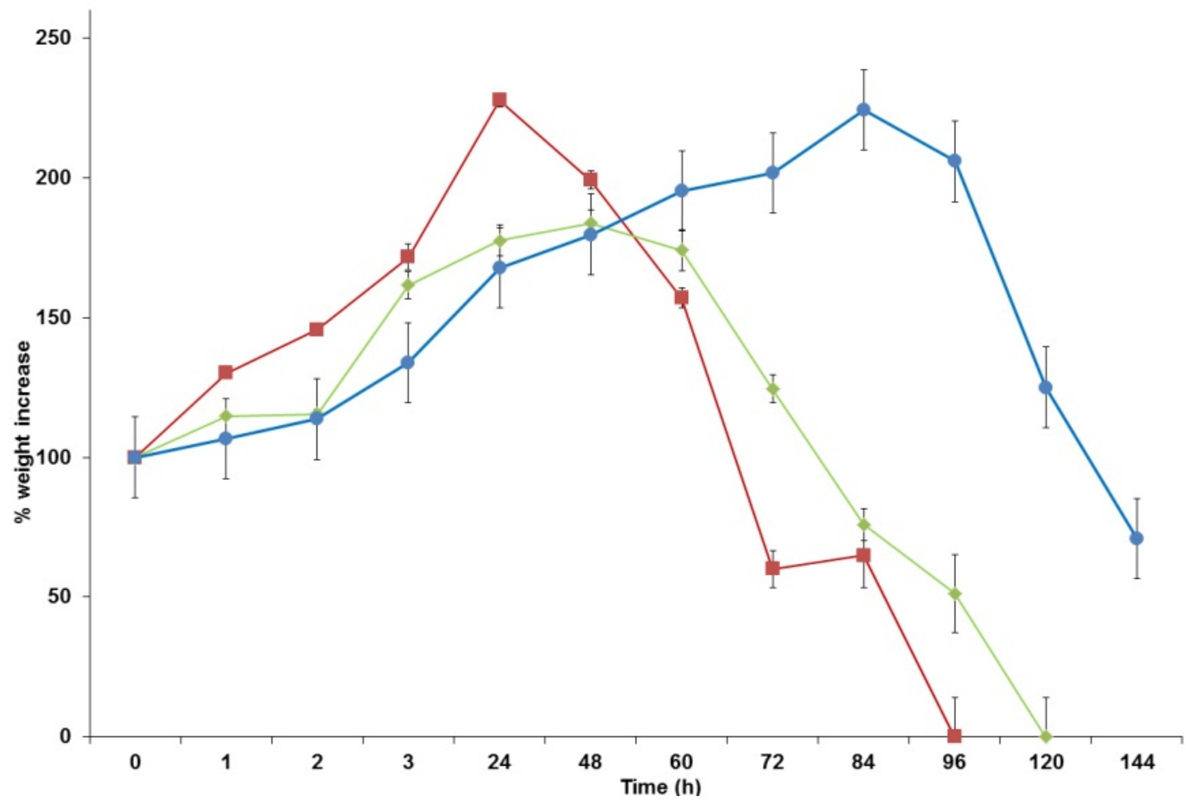

Figure 3

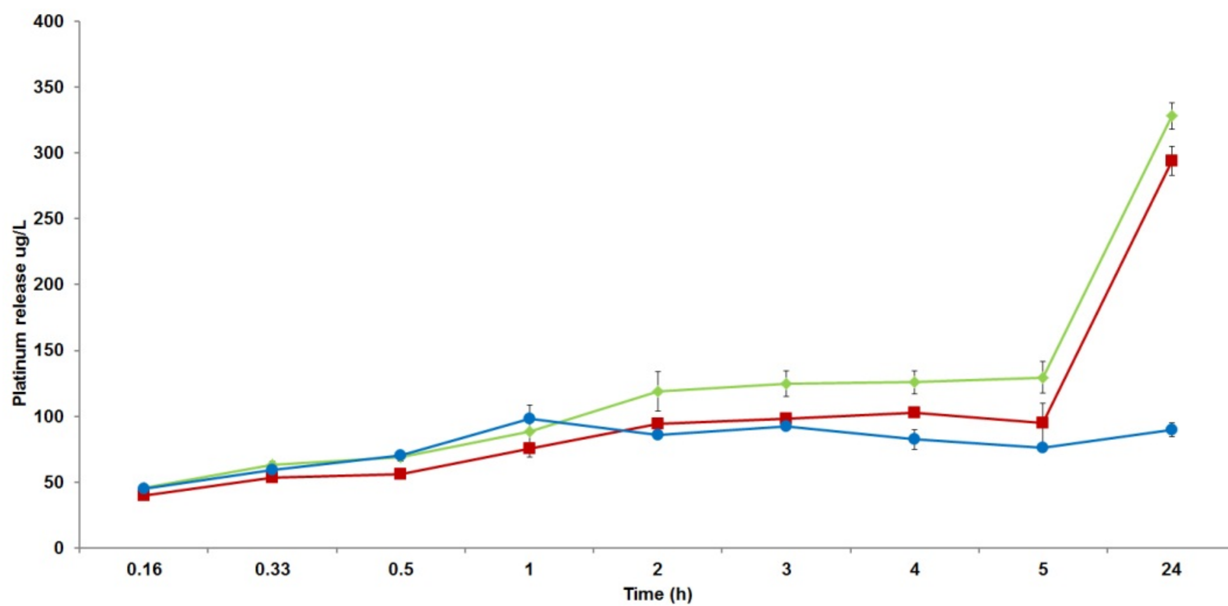

Figure 4

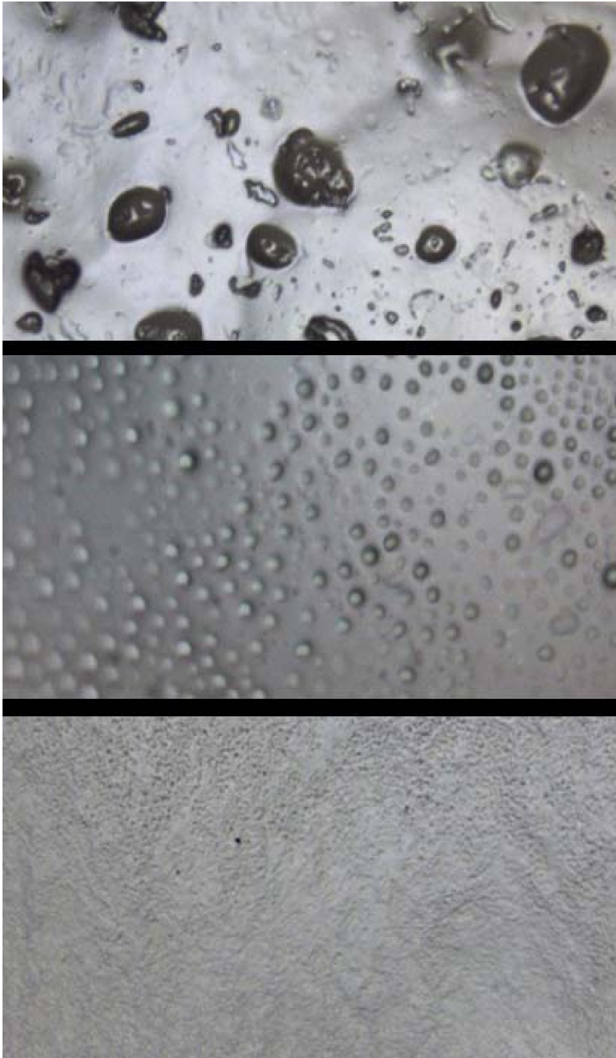

Figure 5

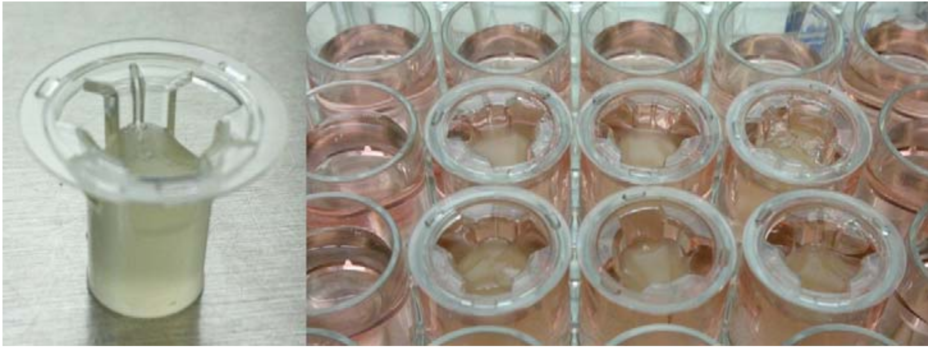

Figure 6

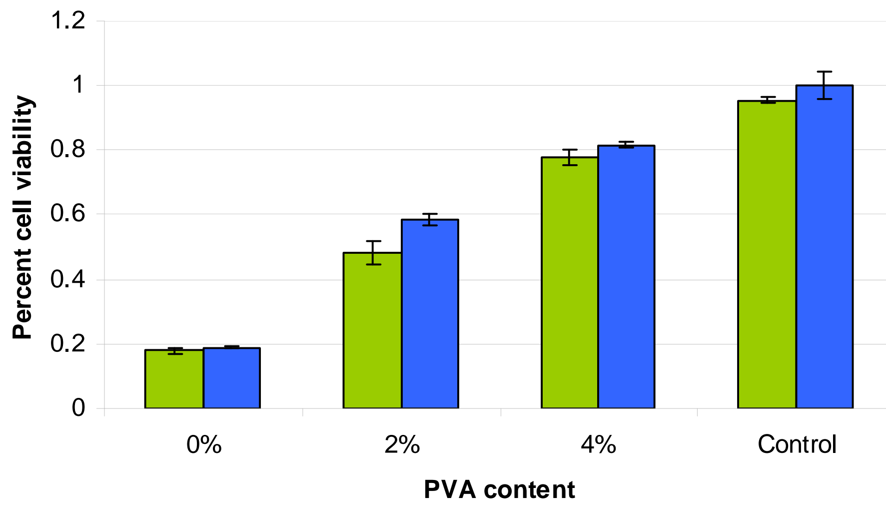

Figure 7

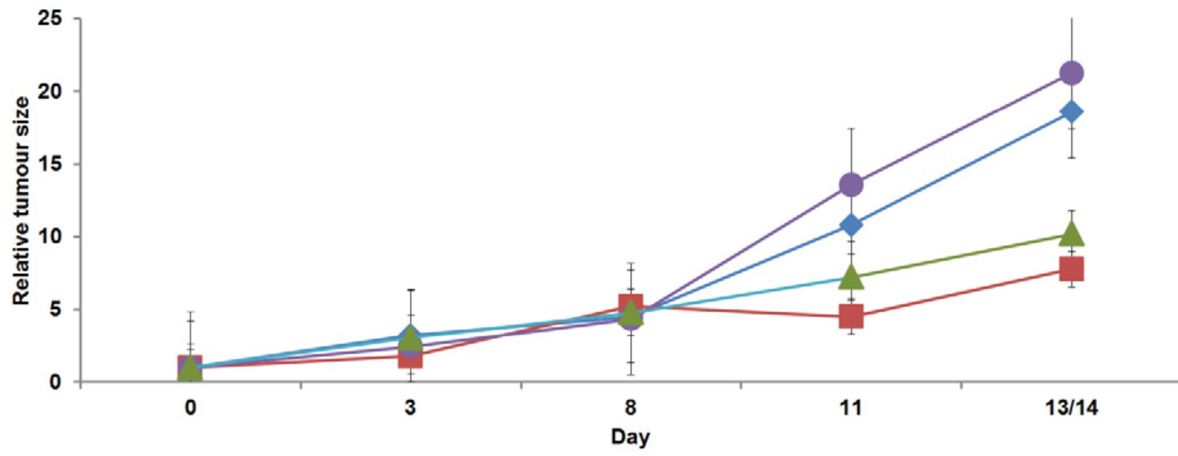

Figure 8