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Development of chitosan, pullulan, and alginate based drug-loaded nano-emulsions as a potential malignant melanoma delivery platform

Ghazal Hatami Fard [a,b,*], Zeynab Moinipoor [b], Salzitsa Anastasova-Ivanova [a], Hafiz M.N. Iqbal [c], Miriam V. Dwek [b], StephenJ. Getting [b], Tajalli Keshavarz [b]

[a] Department of Engineering, Hamlyn Centre for Robotics Surgery, Bessemer Building, South Kensington, Exhibition Road, Imperial College London SW7 2AZ, UK
[b] School of Life Sciences, College of Liberal Arts & Sciences, University of Westminster, 115, New Cavendish Street, London W1W 6UW, UK
[c] Tecnologico de Monterrey, School of Engineering and Sciences, Monterrey 64849, Mexico

A B S T R A C T

Melanoma is the most aggressive form of skin cancer and various treatments have been investigated to treat this disease, but drug resistance remains an important factor in the failure of conventional therapeutics. Here we describe the development, optimisation and characterisation of alginate, chitosan, pullulan, and their combined nano-emulsions as drug delivery platforms for potential application for melanoma. A novel nano-emulsion delivery system was designed and assessed by determining in vitro drug release, cell viability (MTT), cellular apoptosis (ELISA) and confocal microscopy. A comparative analysis of the effect of the nano-emulsions on *BRAF*-mutant melanoma (A375) and keratinocyte (HaCaT) cells was conducted, with the "pullulan-chitosan" nano-emulsion chosen as an approach for melanoma drug delivery. Increased apoptosis induction of melanoma cells was recorded as 90% after 72 h of treatment with doxorubicin-loaded optimal nano-emulsion. Similarly, in the same treatment, the viability of melanoma cells was decreased by 70%. More importantly, A375 cells treated with naïve doxorubicin were 100% viable compared to cells treated with doxorubicin-loaded nano-emulsion which were only 30%viable. Achieved results are indicating the importance of the drug carrier's polymeric combination and the impact of the drug release pattern on the efficiency of the treatment. This offers potential for the abrogation of drug-efflux-related chemo-resistance.

1. Introduction

Malignant melanoma (MM) is a highly lethal skin cancer subtype (Yang et al., 2020) the occurrence of which is increasing worldwide (Moslehi et al., 2018), overall a diagnosis with MM is associated with a 15-20% chance of metastasis over a 5-year period (Garbe et al., 2018). In the UK, from 2016 to 2018, 48 patients were diagnosed with melanoma each day, making melanoma the 5th most common cancer type in the UK (UK, 2022). Risk factors for melanoma include genetic predisposition, fair skin colour and UV exposure (D'Arcy et al., 1984, Markovic et al., 2007). Among these, increased exposure to UV light is the most important initiating factor, alongside factors such as increased alcohol consumption and smoking (Queen, 2017).

The many reasons for treatment failure include the ability of cancerous cells to develop mechanisms to escape apoptosis, this results in continued cell proliferation (Rigon et al., 2015), further factors include poor bioavailability of drugs and inefficient delivery to target cells (Jhaveri and Torchilin, 2016). In common with other solid tumours, the malignancy of melanoma is dependent on the stage at diagnosis. Loco-regional metastasis to the lymph nodes is frequently followed by colonisation of other organs, distant metastasis, and associated acquired mutations rendering treatment less effective (Nguyen and Massagué, 2007, Lohcharoenkal et al., 2018).

Depending on the stage of the disease at diagnosis common treatment procedures involve debulking surgery (removal of the primary tumour), chemotherapy, radiotherapy, targeted therapy, and immunotherapy (MRA, 2007, Mattia et al., 2018). So far, only a few MM-specific therapies have been approved by the FDA, for example, dacarbazine, which has high efficacy against melanoma (Weide et al., 2017, Kandolf Sekulovic et al., 2018) and the development of drug resistance to therapeutics remains an issue associated with low survival rates (Mattia et al., 2018). In this context, targeted therapeutic approaches (Gray-Schopfer et al., 2007) provide an opportunity to increase the effectiveness of treatments for MM (Rapino et al., 2018).

Nano-medicine has emerged as an approach to both deliver drugs and aid in localised imaging of cancer, in an approach known as theragnostic (Wakaskar, 2018). Utilization of nano-sized particles in drug delivery and tissue engineering is increasing of interest due to attributes such as enhanced permeation and retention, controlled delivery, co-delivery of larger drugs and small molecules, relatively easy

* Corresponding author.
E-mail address: G.hatami-fard@ic.ac.uk (G.H. Fard).

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preparation, improved drug stability and the provision of a smart platform for drug delivery (Farokhzad and Langer, 2009, Rigon et al., 2015). Nano-emulsions, two-phase systems containing oil and water that confer solubility to non-soluble drugs, have been proposed as suitable tools for cancer drug delivery. Nano-emulsions can be functionalised to enable the specific targeting of affected tissues (Mahato, 2017) and enhanced therapeutic encapsulation capacity (Ferrari, 2005, Khatri et al., 2013). Preparation techniques for nano-emulsions differ depending on the materials used and their proposed application (Salata, 2004).

Presenting unique characteristics, polysaccharides are among the suitable polymers for melanoma drug delivery and have been successfully used in cancer drug delivery systems (Yang et al., 2016). Chitosan is a natural polymer comprising of a polysaccharide backbone, and whilst barely soluble in water at non-acidic pH (Ye et al., 2018, Ibrahim et al., 2020), it offers several potential advantages as a transdermal drug delivery system (Asefi et al., 2018), including pH sensitivity, extracellular matrix-like structure, fast degradation under physiological conditions, non-toxic and antibacterial activity (Nawaz and Wong, 2018, Carter et al., 2019, Salama et al., 2022, Ali et al., 2022, Abou-Okeil et al., 2022). These characteristics make it a good choice for the development of a cancer drug delivery vehicle (Carter et al., 2019), making it one of the most advantageous polymers to be used in transdermal drug delivery systems (Nawaz and Wong, 2018, Carter et al., 2019, Luo et al., 2021). Alginate, a naturally occurring polymer, is water-soluble, non-toxic, and biodegradable and has been used and assessed as a potential polymer for cancer drug delivery (Jayapal and Dhanaraj, 2017, Shen et al., 2018). Pullulan, a natural hydrophilic, non-toxic, biodegradable polysaccharide has also been used in drug delivery preparations. Pullulan has a fast rate of degradation and this feature has resulted in its proposed use for chemotherapy drug encapsulation and modification of delivery systems (Fan et al., Li et al., 2019). Each of these three polymers has specific properties and has been evaluated in studies focused on cancer drug delivery formulations individually.

The current research investigates the potential of the polysaccharides above as nano-emulsions for more effective drug delivery to A375 cells. The aim of this study was to assess the effect of uptake and release of therapeutics on melanoma cell behaviour. The capability of the nano-emulsions to induce apoptosis to A375 was evaluated. The polymers pullulan, chitosan, and alginate were evaluated as a combined cancer drug delivery system. The formulations were evaluated (i) by loading with the fluorescent dye coumarin-6 and (ii) by loading with doxorubicin a chemotherapeutic drug used for the treatment of solid tumours, including melanoma (Capanema et al., 2018a), this enabled particle tracking and drug release (Miao et al., 2015). The nano-emulsion providing optimal coumarin-6 and doxorubicin release was identified and used with *BRAF*-mutant A375 melanoma and HaCaT cells. Subsequently, doxorubicin was encapsulated in the optimised formulation and cellular uptake by keratinocytes and malignant melanoma cells was assessed.

2. Materials and methods

2.1. Materials and reagents

Chitosan (medium molecular weight, 75–85% deacetylated chitin), Glacial acetic acid (HPLC grade), Ethanol (HPLC grade), acetonitrile (HPLC grade), Coumarin-6 (98%), pullulan (Aureobasidium pullulans), sodium alginate, genipin, doxorubicin (HPLC grade), Dulbecco's Modified Eagle's Medium (DMEM) high glucose, Dulbecco's phosphate-buffered saline (DPBS) (Modified, without calcium chloride and magnesium chloride, liquid, sterile-filtered), and trypsin-EDTA solution were purchased from Sigma Aldrich (Poole, Dorset, U.K.).

Foetal bovine serum (FBS, One-shot format, heat-inactivated and filtered) was purchased from Thermo-Fischer scientific (UK). Dialysis tubing 12 molecular weight cut-off (MWCO) was purchased from Spectrum Labs, Inc (US). A375 (BRAF-mutant malignant melanoma) and HaCaT (human immortalized keratinocyte) cell lines were obtained from ATCC and UCL Medical school Royal free hospital) respectively.

2.2. Preparation of drug-loaded polysaccharide-based nano-emulsions

Nano-emulsions were prepared using chitosan (C), pullulan (Pl), and alginate (Al) solutions with w/w ratios of 2, 4, 6, 8, 10, 12, and 14 mg/ml as follows. Chitosan powder was solubilised in ultrapure water containing the same ratio of glacial acetic acid to chitosan and stirred for 24 hours at room temperature to obtain a homogenous solution. Tween-80, at a ratio of 5:1 v/v to chitosan, was added to glycerol (oil phase of the nano-emulsion) and mixed for 2 hours. This method was used with slight modification, which includes utilising glycerine as the main oil component (with an increased ratio) (Saberi et al., 2013). The coumarin-6 solution, (50 µl, 1 mg/ml) was added to the Tween-80/glycerol and mixed for 1 hour at room temperature, the chitosan solution was added and mixed using a magnetic stirrer (Hanna Instruments Deutschland GmbH) for a further 2 hours. The mixture was sonicated using a high-energy homogeniser (Phillip Harris Scientific, UK) for two rounds of 20 minutes on ice (ice replaced every 5 minutes), to obtain coumarin-6 loaded nano-emulsions. Finally, the nano-emulsion was dialysed against phosphate-buffered saline (PBS) to remove free coumarin-6 particles. The pH of the nano-emulsion samples was adjusted to 7.4 using 1 M sodium hydroxide. The same procedure was used for doxorubicin encapsulation, however, doxorubicin concentration in the nano-emulsions was 1µg/ml (Norouzi et al., 2020). Alginate and pullulan nano-emulsions were prepared in the same manner; however, both were solubilised in ultrapure water, pH 7. Chitosan, alginate, and pullulan nano-emulsions are described as chitosan nano-emulsion (NEC), alginate nano-emulsion (NEAl), and pullulan nano-emulsion (NEPl) in the text, respectively.

Based on the *in vitro* coumarin-6 release profiles, optimal nano-emulsion from each polymer group was selected for blending. Accordingly, chitosan-pullulan, pullulan-alginate, and chitosan-alginate blended nano-emulsions were prepared using different w/w ratios (1:2, 2:1, 1:1), whilst Genipin was used as a crosslinker. The crosslinking procedure was also applied to three previously selected non-blended formulations, chitosan, pullulan, and alginate (as control nano-emulsions for blended nano-emulsions). All prepared blends were loaded with coumarin-6 and doxorubicin separately, whilst fifteen blended nano-emulsions were prepared using the same procedure. UV sterilisation was applied over two 20-minute cycles before cell treatments. Coumarin-6 and doxorubicin release tests were also conducted for 15 blended nano-emulsions, under the same conditions as stated above. The same preparation and drug loading procedure were applied to prepare doxorubicin-loaded nano-emulsions. Naïve coumarin-6 and doxorubicin were used as positive controls in the treatments and calculations. Table 1 represents abbreviations used for nano-emulsions and details of polymer concentrations.

2.3. Nano-emulsion characterisation

Nano-emulsions were characterized by size, concentration, and encapsulation efficiency. To obtain the encapsulation efficiency within

Table 1
The in-house developed nano-emulsion samples.

Chitosan	Polymer ratio (w/v%)	Pullulan	Polymer ratio(w/v%)	Alginate	Polymer ratio (w/v%)
NEC1	0.2	NEPl1	0.2	NEAl1	0.2
NEC2	0.4	NEPl2	0.4	NEAl2	0.4
NEC3	0.6	NEPl3	0.6	NEAl3	0.6
NEC4	0.8	NEPl4	0.8	NEAl4	0.8
NEC5	1.0	NEPl5	1.0	NEAl5	1.0
NEC6	1.2	NEPl6	1.2	NEAl6	1.2
NEC7	1.4	NEPl7	1.4	NEAl7	1.4

nano-emulsion particles, coumarin-6 loaded nano-emulsion was dialyzed against phosphate-buffered saline (PBS) and pH was adjusted to 7.4 using HCl. To calculate the drug encapsulation efficiency, 1 ml of each dialysis sample was dialysed (12–14 kDa cut off Spectrum Laboratories, Inc.) against PBS buffer. The amount of free coumarin-6 in PBS was quantified using a spectrophotometer (Novaspec II, UK) reading at 450 nm, and encapsulation efficiency was calculated using the following equation:

$$Encapsulation\ efficiency = \frac{total\ drug\ loaded - free\ drug}{total\ drug\ loaded} * 100$$

The same procedure was applied for the measurement of doxorubicin encapsulation efficiency and data obtained at 457 nm (Motlagh et al., 2016).

To identify the size distribution of nano-emulsion particles, the samples were analysed using a particle tracking analyser (Nano Sight nano-particle tracking analyser, Malvern UK) enabling particle sizes and particle concentrations of the formulations to be obtained. Simply, 1 ml of doxorubicin-loaded nano-emulsions (dilution factor 1:100 in PBS) was loaded in the test cuvette and imaged with the Nano Sight particle tracking analyser.

The shape of the nano-emulsions was imaged using SEM (Lyra3 Tescan). For this purpose, nano-emulsion was diluted 10 times using PBS. Fifty microliters of the diluted nano-emulsion were placed on the probe coated with a carbon patch. The probe was left in a vacuum chamber to dry. The stubs were examined under scanning electron microscopy (SEM) at an accelerating voltage of 4 kV and magnification of 689×. The specific regions of the samples were d accordingly.

2.4. In-vitro drug release experiment

The release of coumarin-6 and doxorubicin from nano-emulsion preparations were assessed before tracking the cellular uptake of drug-carrying preparations by dialysis (Wallenwein et al., 2019, Eley et al., 2004, Yu et al., 2019). Nano-emulsion samples of 1 ml were loaded into dialysis tubing (12-14 kDa cut-off) and dialysed against 40 ml of PBS (pH 7.4) on an orbital shaker at 55 rpm, 37°C for 21 days in the dark. The dialysate was sampled at a range of time points (24, 72, 168, 216, 336, 384, 432, and 480 hours) and replaced by the same volume of PBS. The optical density of the sampled dialysate was read at 450 nm and 479 nm for coumarin-6 and doxorubicin respectively, using a UV-spectrophotometer.

2.5. Cell culture

A375, a *BRAF* mutant malignant melanoma primary cell line (derived from a 54-year-old female, ATCc catalog Number CRL-1619), and HaCaT cells a human epithelial keratinocyte primary cell line was used in this study. The cells were cultured in T-75 flasks with DMEM containing 10% v/v FBS. Cells were incubated at 37 °C and in 5% v/v CO_2 in a humidified atmosphere. Once cells reached 80% confluency, they were lifted with trypsin/EDTA and seeded in 6-well plates (10^6 cells per well), allowed to adhere for 8 h and used for confocal microscopy and 96 well plates (10^5 cells per well) used for MTT assay. All experiments were conducted within 14–16 passages.

2.6. Cell viability assay

MTT assay was conducted to investigate the cytotoxicity of the doxorubicin-loaded formulations to the cell cultures. MTT dye, 3-(4, 5-dimethylthiazol-2-yl)−2, 5-diphenyltetrazolium bromide, at a concentration of 5 mg/ml in serum-free DMEM was prepared before the experiment. MTT solution was added to each well of the treated cells equivalent to 10% of the culture medium. The plate containing the MTT dye was incubated for 4 h at 37 °C and 5% v/v CO_2, the cell culture medium was then removed and 200 μl of DMSO was added to each well. The plate was incubated at room temperature overnight and cytotoxicity was defined by a 96 well plate-reader at 570 nm. Cells without treatment were used as a positive control in the calculations.

2.7. Confocal microscopy

Cells (A375/ HaCaT) seeded into 6-well plates were exposed to coumarin-6 and doxorubicin-loaded nano-emulsion treatments (10 μl/ml). After 72 h of treatment, cells were washed with PBS twice and fixed using 4% v/v formaldehyde. PBS was added to each well and the cells were imaged using confocal microscopy (Leica TCS SP2, Germany). Confocal microscopy images were obtained at 488–530 nm for doxorubicin and 430–485 nm for coumarin-6.

2.8. Cellular apoptosis measurement

Cellular apoptosis of treated cells was studied using DNA fragmentation ELISA assay according to the manufacturer's protocol (Hassan et al., 2016). This assay was used to investigate the ability of the doxorubicin-loaded formulation to induce apoptosis on A375 melanoma as well as HaCaT keratinocyte cells. Cell apoptosis was detected according to the manufacturer's protocol using cellular fragmentation ELISA kit (11585045001) (Sigma Aldrich, Dorset, UK). Briefly, cells reached 80% confluency and were harvested using trypsin-EDTA. Following detachment, 5 ml of the cell media was added to the flask, the cell suspension was pipetted out and centrifuged for 5 minutes at 15, 000 rpm. The supernatant was removed, and the cell pellet was re-suspended in fresh media containing BrdU labelling reagent to a final concentration of 10 μM and cell suspension incubated overnight at 37°C and 5% CO_2. Following incubation, the BrdU labelled cell suspension was centrifuged for 10 minutes at 250 *g*, supernatant decanted and labelled cells were re-suspended in fresh media. BrdU labelled cells were seeded into duplicate wells of round-bottom 96-well plates to a final concentration of 1×10^5 cells/ml. Cells were treated with 10 μl/ml of formulations and incubated for 4, 24, 48, and 72 hours, following incubation plates were centrifuged for 10 minutes at 250 *g* and the supernatant was removed. Cells were lysed for over 30 minutes at 37°C by adding incubation solution (supplied by the manufacturer) and plates centrifuged at 2000 g for 10 minutes, supernatants were collected from each well and then added to pre-coated plates for ELISA photometric measurements using 96 well plate reader at 370 nm.

2.9. Statistical analysis

All tests were performed as 3 analytical replicates and 6 biological replicates. The results were analysed using One-way ANOVA, Tukey's comparison test, in Graphpad Prism (version 8.4.3).

3. Results and discussion

3.1. Characterisation of optimal pullulan-chitosan nano-emulsion

Optimisation studies with a range of nano-emulsion blends were undertaken as described above (data not shown). The pullulan-chitosan nano-emulsion NEPl2-CS 1:2 increased the delivery capacity (encapsulation efficiency) to 87% for both doxorubicin and coumarin-6. Based on these results and the viability assay and uptake experiments, this nano-emulsion was characterised further. Fig. 1A shows the mean average particle size ± SD was115 nm. Fig. 1B shows the oil in water nano-emulsion imaged using scanning electron microscopy (SEM); the particles were spherical, but their size distribution was not homogenous, consistent with the results obtained from the Nano-particle Tracking Analyser (NTA).

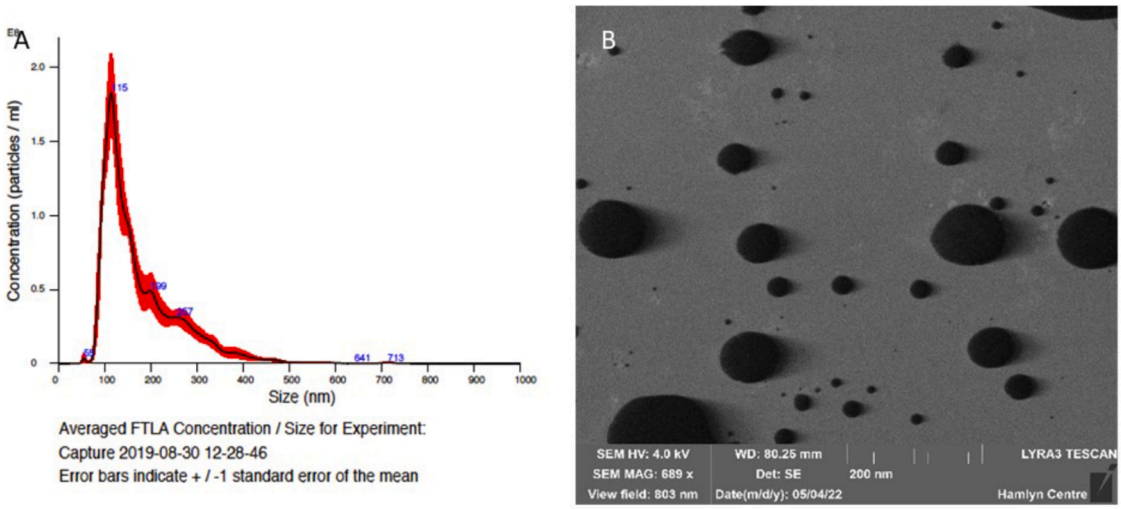

Fig. 1. Characterization of nano-emulsion NEPl2-CS 1:2. A) Doxorubicin-loaded nano-emulsion size and concentration distribution. The data were obtained using Nano-particle Tracking Analyser (NTA). Three sample injections were performed for each nano-emulsion sample. B) SEM imaging of 10X diluted nano-emulsion.

3.2. Evaluation of coumarin-6 and doxorubicin in vitro release from blended and non-blended nano-emulsions

There are several causes for the failure of conventional chemotherapeutics in melanoma. These include lack of metabolic activation of the drug, increased drug flux to the tumour, epigenetics, altered paracrine signalling, apoptosis pathway alteration, and mesenchymal-epithelial transition, all of which may lead to drug resistance (Housman et al., 2014). Furthermore, the mechanisms underlying drug resistance reflect the genetic landscape of cancer and the host immune response. Malignant melanoma cells have been reported to have increased levels of P-glycoprotein and multidrug resistance-associated proteins (Bhatnagar et al., 2018). Over the course of chemotherapy treatment the levels of such proteins increase, thus, even with increased doses of therapeutics, melanoma cells develop resistance to apoptosis (Penson et al., 2004, Colone et al., 2008). Hence, high, or low drug delivery efflux is an important triggering factor for the emergence of drug resistance.

Whilst targeted therapies and controlled delivery of therapeutics have been achieved successfully to overcome resistance and initiate apoptosis for various cancer types (Naves et al., 2017, Plch et al., 2019), there remains a need to identify new modalities of potential application for melanoma.

In this context, controlled delivery of therapeutics via polysaccharide-based nano-particles has been investigated as an approach for the delivery of therapeutics to melanoma cells (Mundra et al., 2015, Posocco et al., 2015). The main aim of the current study was to provide a novel nano-emulsion, capable of uptake into melanoma cells while avoiding healthy skin cells and highlighted by successful induction of apoptosis in melanoma cells. The mentioned features of the nano-emulsion were investigated to manipulate drug-efflux and investigate its consequences on the reduction of melanoma cell viability *in vitro*.

The *in vitro* release profiles of coumarin-6 from pullulan, chitosan, and alginate nano-emulsions were recorded over a period of 21 days. The individual and blended polymer nano-emulsions exhibited different release profiles with no statistically significant differences evident. All nano-emulsions showed limited release of coumarin-6 during the 21-day period, compared to naïve-non-encapsulated drug.

The criteria used for subsequent nano-emulsion selection was the lowest initial burst followed by a controlled release profile of the encapsulated drug, Figure 2, and Figure 3. A single nano-emulsion from each polymer group was chosen and mixed using 2:1, 1:2 and 1:1 ratios Consequently, 15 blended nano-emulsion were prepared and loaded with doxorubicin, and coumarin-6, separately. The release test was repeated for all the blended nano-emulsions. Adopting this approach, overall, the release profiles were improved compared to non-blended nano-emulsions. The blend comprising pullulan and chitosan at a ratio of 1:2 respectively exhibited the optimum release profile, Figure 4.

This study aimed to identify a polymer with a stable and controlled pattern of DOX release, Figures 2, 3 and 4 show that the polymer-blends limited the drug efflux. Amongst the range of blended-nano-emulsions assessed the most suitable candidate (as determined by the controlled drug release profile) for drug delivery to A375 cells was the pullulan-chitosan 1:2 nano-emulsion. The polymer surface charges were also considered as a potential factor affecting drug release rate and consequently the stability of the nano-emulsion. Since the optimal nano-

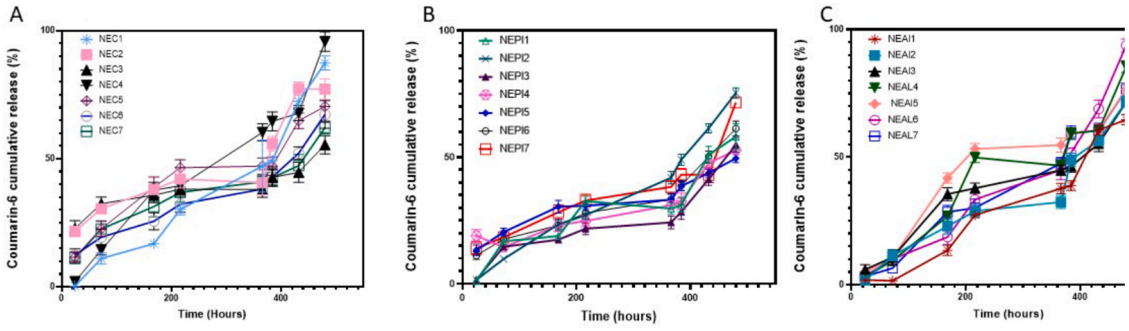

Fig. 2. Coumarin-6 release profiles over a 504-hour (21 day) period from A) Chitosan nano-emulsions, B) Pullulan nano-emulsion, and C) Alginate nano-emulsions ($n = 3$).

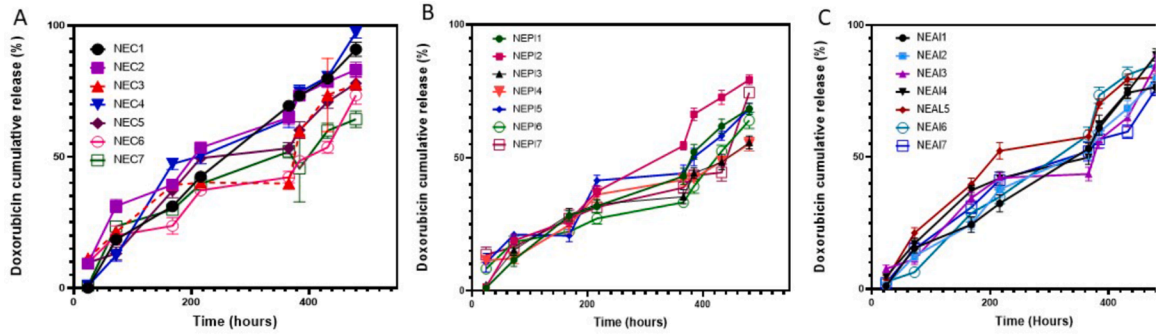

Fig. 3. Doxorubicin release profiles over a 504-hour (21 day) period from A) Chitosan nano-emulsions, B) Pullulan nano-emulsion, and C) Alginate nano-emulsions ($n = 3$).

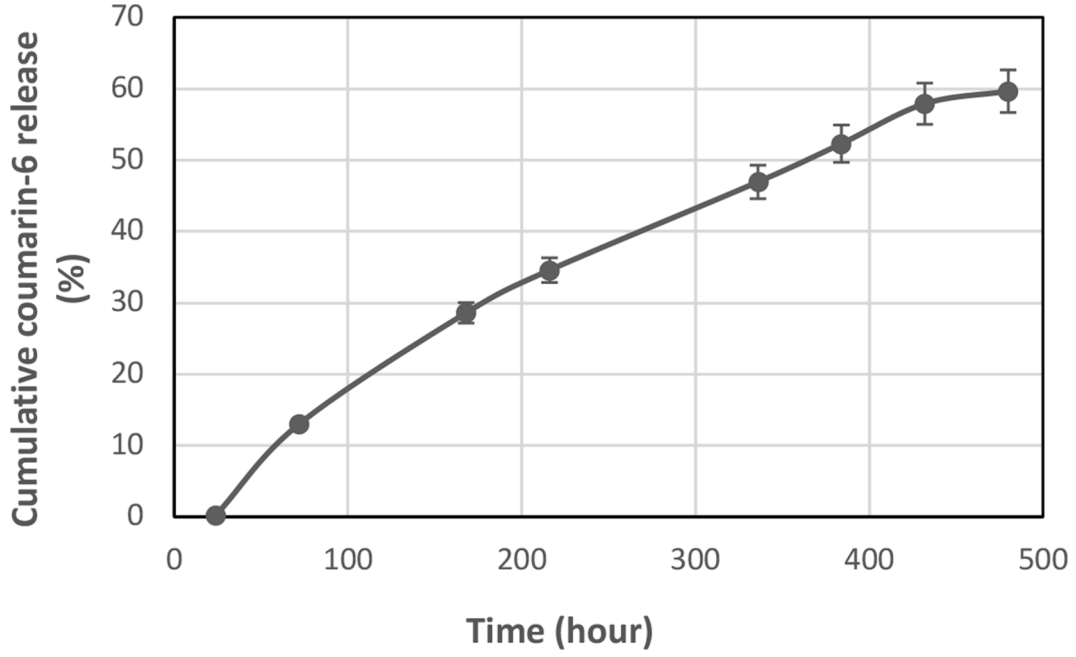

Fig. 4. In vitro release profile of Pullulan-chitosan (1:2 ratio) blended nano-emulsion ($n = 3$). The release profile was obtained over a 504-hour (21 days) period at 37 °C.

emulsion was a combination of chitosan and pullulan successful uptake of the nano-emulsion could also be an indication of the surface charge effect. Also in this case the effect of positively charged chitosan will be dominant due to a higher weight ratio (Bruinsmann, 2019).

3.3. Evaluation of cell viability (MTT assay)

The MTT cytotoxicity assay was conducted to investigate the effect of 15 non-loaded blended nano-emulsions on A375 (melanoma) and HaCaT (Keratinocytes, control) cell lines. The experiments were performed in six independent experiments and the mean average was used to calculate the percentage of cellular viability.

The aim was to provide nano-emulsions that were minimal in their cytotoxicity towards control cells (HaCaT) and maximal against melanoma cells (A375). All blended nano-emulsions were used as treatments.

Figs. 5 and 6 show the viability of cells treated with three different nano-emulsion concentrations. The viability of keratinocyte cells ranged from 75 to 106% (NEPl2-Al 1:2 and NEPl2-Cs 1:2), 43 to 85% (NEAl2-Cs 2:1 and NEC1-Al 2:1) and 47 to 80% (NEAl2-Cs 2:1 and NEPl2-Cs 1:1) for treatment doses of 10, 20, and 30 μl/ml respectively compared to untreated control. Melanoma cell viabilities ranged from 70 to 107% (NEC1 and NEPl2-Cs 1:2), 56.14 to 87% (NEAl2-Cs 2:1 and NEC1-Al 2:1) and 28 to 96% (NEAl2-Cs 1:1 and NEPl2-Al 1:1) for treatment doses of 10, 20 and 30 μl/ml respectively compared to untreated control. This set of experiments aimed to identify the optimal dose and determine which of the non-loaded nano-emulsions did not decrease keratinocyte viability whilst decreasing the melanoma cell viability. Accordingly, the nano-emulsion treatment dose of 10 μl/ml was chosen.

Increasing the treatment dose to 20 and 30 μl/ml, significantly decreased the viability of both A375 and HaCaT cells to a maximum of 45%, for a few treatments. Hence, the lowest dose was chosen for subsequent treatments. A good example was sample NEPL2-Cs 1:2 which had a significant desirable effect compared to other treatments. This nano-emulsion had no significant toxic effect on HaCaT cells at a 10 μl/ml dose (100% viable), however, the viability decreased by approximately 40% after increasing the treatment dose three times.

Nano-emulsion formulations NEC1-Al 2:1, NEAl2-Cs 1:1, NEPl2-Cs 2:1, NEPl2-Cs 1:2, NEC1-Pl 1:1, NEC1-Pl 2:1, NEPl2-Al 2:1, NEPl2-Al 2:1 and NEPl2-Al 1:1 were chosen to investigate the effect of doxorubicin encapsulation (Figs. 5 and 6). Subsequently, the doxorubicin was encapsulated in the nine most effective nano-emulsions. The toxicity of the doxorubicin-loaded and blank nano-emulsions was compared to the toxicity of naïve doxorubicin (Fig. 7) and untreated A375 cells. These experiments aimed to determine the optimal non-loaded nano-emulsions which did not negatively affect HaCaT cell viability after 72 h of exposure. This approach was used to narrow down the identification of

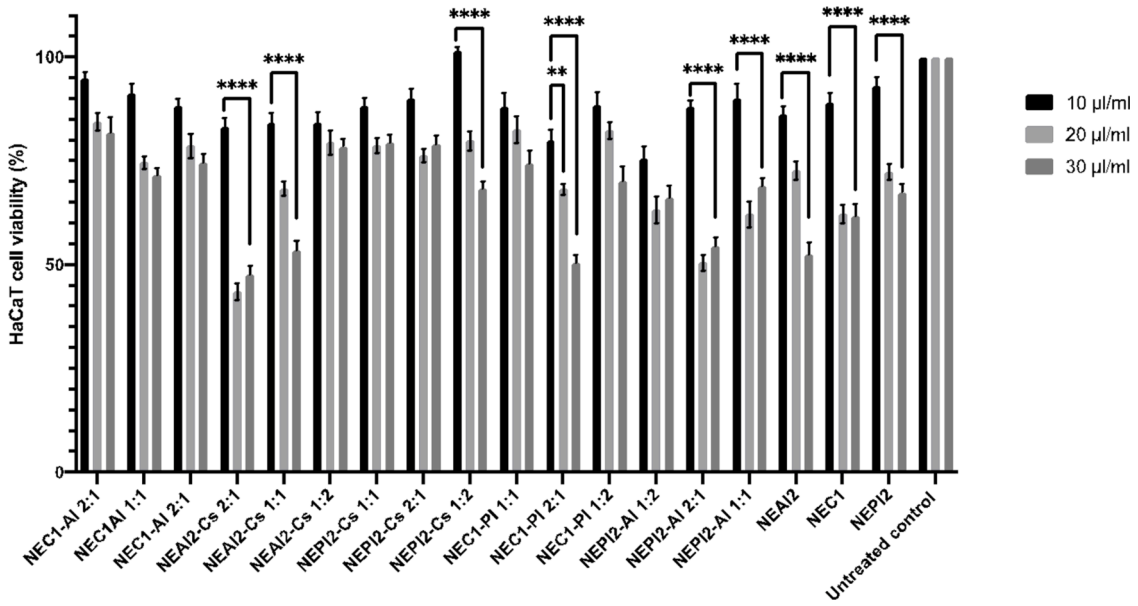

Fig. 5. Viability screening keratinocyte (HaCaT) cells treated with non-loaded nano-emulsions. Bars represent the mean value of 6 independent experiments ±SD error. The data were analysed with two-way ANOVA Tukey's comparison test, **** is indicating $P<0.0001$, *** $P<0.001$, ** $P<0.01$, and * $P<0.05$.

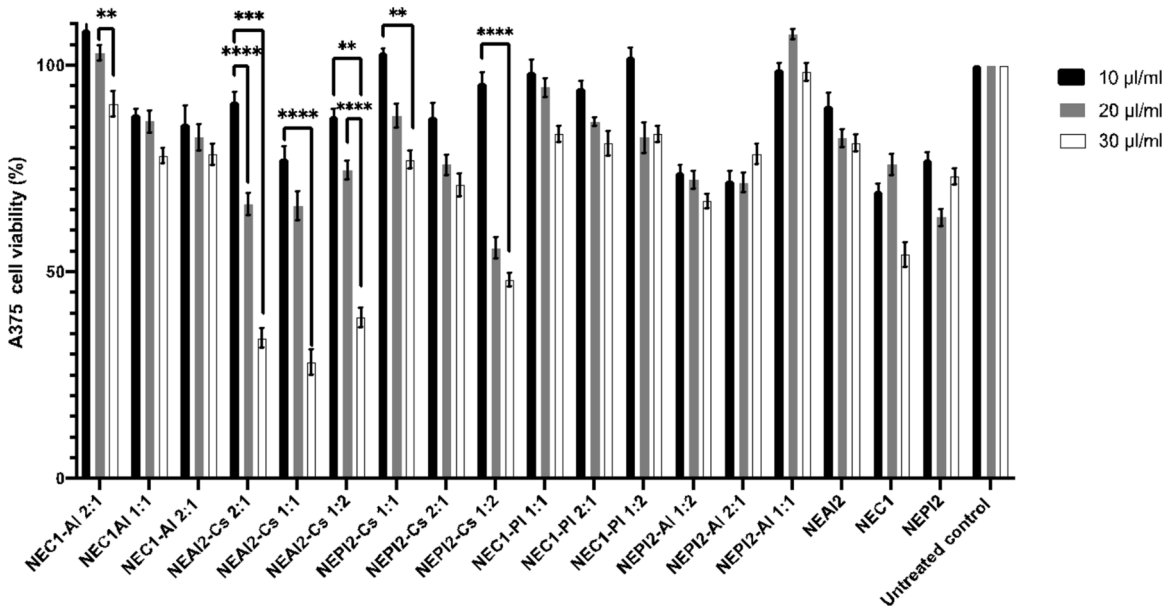

Fig. 6. Viability screening Melanoma (A375) cells treated with non-loaded nano-emulsions. Bars represent the mean value of 6 independent experiments ±SD error. The data were analysed with two-way ANOVA Tukey's comparison test, **** $P<0.0001$, *** $P<0.001$, ** $P<0.01$, and * $P<0.05$.

the samples to the ones decreasing A375 viability whilst maintaining HaCaT viability. Some treatments resulted in a significant reduction in A375 cell viability while the viability of HaCaT cells remained high compared to untreated control cells. A good example is sample NEPL2-Cs 1:2 which had a significant desirable effect compared to other treatments.

Fig. 7, shows that A375 cells in presence of doxorubicin treatment have the highest viability amongst all chosen treatments. Importantly, doxorubicin-loaded formulations resulted in reduced viability of melanoma cells. NEPl2-Cs 1:2 and NEC1-Pl 2:1 nano-emulsions showed the highest viabilities compared to the non-loaded nano-emulsion. Yet, the same treatments (NEPl2-Cs 1:2 and NEC1-Pl 2:1) demonstrated decreased viability in presence of doxorubicin-loaded nano-emulsion. Encapsulation of doxorubicin in nano-emulsions resulted in decreased A375 cell viability compared to the untreated control. Amongst all the nano-emulsions the chitosan-pullulan combination showed the most favourable effect in terms of cell penetration and controlled drug release. Also, compared to A375 cells, chitosan-pullulan nano-emulsion had lower toxicity to HaCaT cells. This indicates that the chitosan-pullulan nano-emulsion has more affinity to A375 cells than HaCaT.

According to the viability tests, uptake of the polysaccharide-based nano-emulsion to A375 cells was slightly increased than the uptake by HaCaT. Furthermore, the viability of the A375 cells treated with non-loaded nano-emulsion was higher than those cells treated with doxorubicin-loaded nano-emulsion and naïve doxorubicin.

This might be due to the increased efficacy of the encapsulated doxorubicin, or enzymatic degradation of it within the cell cytosol. This result can also be an indication of adjusted doxorubicin delivery efflux to the A375 cells.

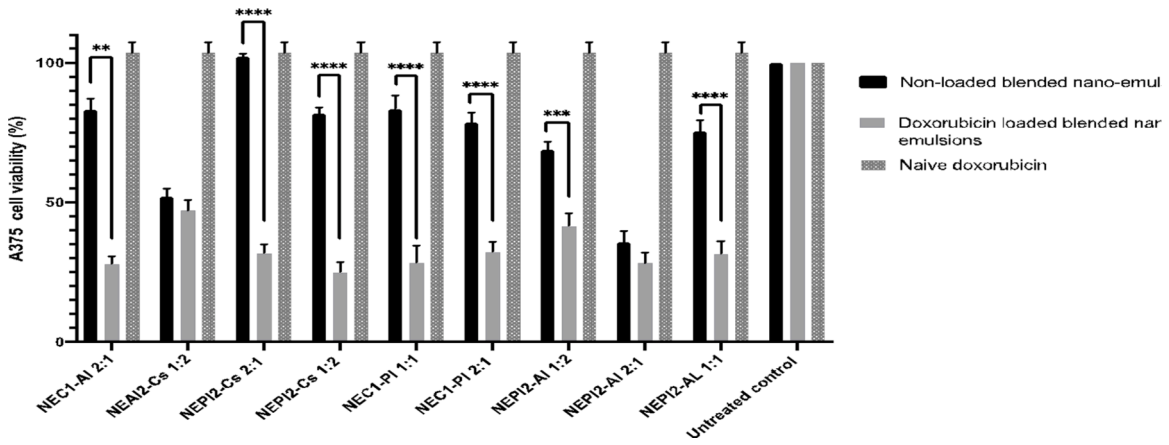

Fig. 7. The A375 cells were treated with non-loaded nano-emulsions, doxorubicin-loaded nano-emulsion, and naïve doxorubicin ($n = 6$). The bars are mean ± SD analysed with two-way Anova. The experiment (non-loaded blended nano-emulsion, doxorubicin-loaded blended nano-emulsions, and naïve doxorubicin) was conducted to compare the effect of three treatments on the A375 cell line. The data were analysed with two-way ANOVA Tukey's comparison test, ****$P<0.0001$, *** $P<0.001$, ** $P<0.01$, and * $P<0.05$.

3.4. Cellular uptake

To observe the uptake of the optimal nano-emulsion particles *in vitro*, the treated A375 cells were imaged using confocal microscopy (Wang et al., 2012, Drasler et al., 2017). The treatments (doxorubicin-loaded nano-emulsion, coumarin-6-loaded nano-emulsion, naïve doxorubicin, and naïve coumarin-6) were applied to A375 cells for 72 hours. **Error! Reference source not found.**8 indicates the uptake of nano-emulsion particles compared to naïve drugs in A375 cells.

Figure 8 shows the effect of the uptake of encapsulated and naïve drugs, based on fluorescent intensity. As the images show, the encapsulation of both doxorubicin and coumarin-6 led to better uptake in

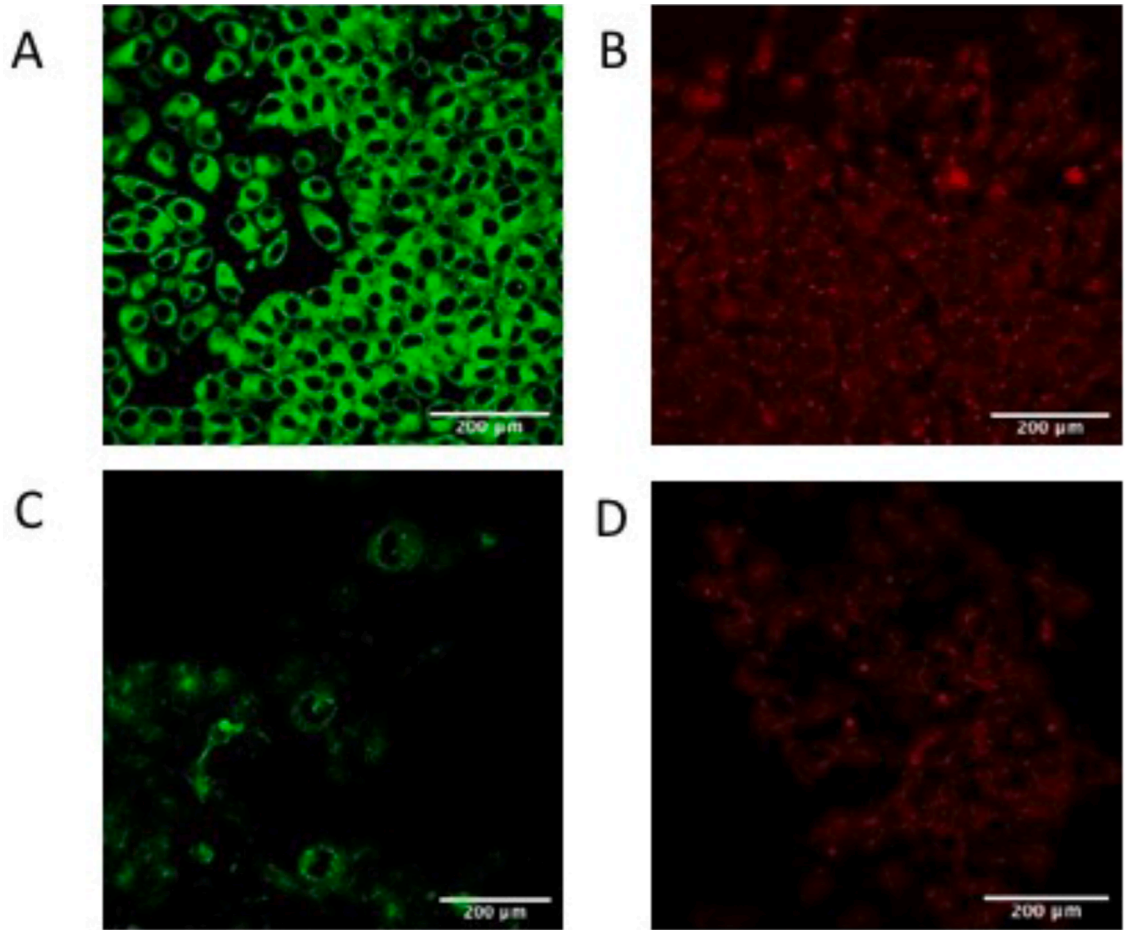

Fig. 8. Cellular uptake of (A) encapsulated coumarin-6, (C) naïve coumarin-6 (B) encapsulated doxorubicin and (D) naïve doxorubicin in A375. The representative images are indicating increased uptake of the drug-containing particles in A and B figures, compared to C and D. the fluorescence intensity for both A and B images was significantly higher than in C and D. Cells were incubated and treated in similar conditions (scale bar: 200 µm).

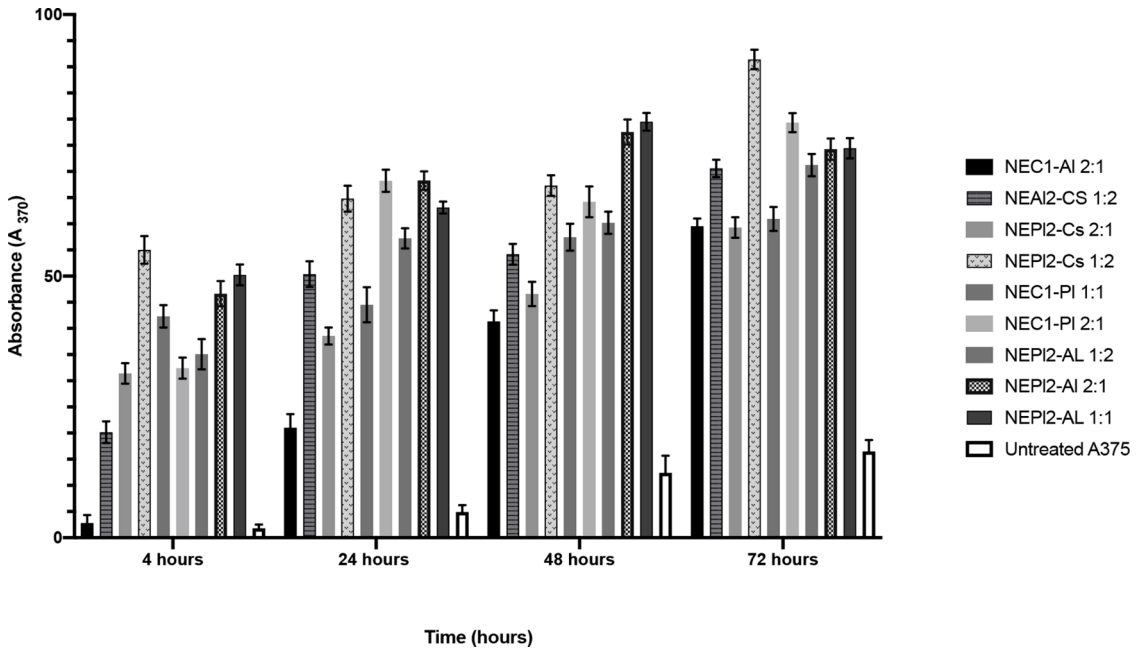

Fig. 9. Induced apoptosis trend by doxorubicin-loaded nano-emulsions to A375 cells, measured by DNA fragmentation ELISA. The A375 cells were treated with doxorubicin-loaded top-nine blended nano-emulsions for periods of 4, 24, 48, and 72 h to quantify the apoptosis induction ability of doxorubicin-loaded nano-emulsions ($n = 3$). According to the protocol, absorbence is recorded as apoptosis induction ability. Bars indicate mean values ±SD error bars. The data were analysed using one-way ANOVA.

A375 cells. This follows with the enhanced activity of the drugs toward decreasing A375 viability compared to untreated A375 or Naïve drug-treated A375 viability.

3.5. Cellular apoptosis

To confirm the effect of drug-loaded nano-emulsions on melanoma cells, cellular apoptosis was measured using a DNA fragmentation ELISA kit. The test was applied to treated melanoma cells with doxorubicin-loaded nano-emulsions showing an increase in apoptosis in A375 cells over 4 - 72 h. Naïve doxorubicin was used as the control (Fig. 9). The most consistent apoptosis induction was observed with the formulation NEPl2-Cs 1:2Based on these experiments NEPl2-Cs 1:2 was chosen as the optimised nano-emulsion. The main reason for this was the consistent increase in the apoptosis induction, over 72 h. Whereas the other nano-emulsion samples had lower performance compared to NEPl2-Cs 1:2.

4. Conclusion

This study a combination of polymers was used to investigate the effect of positive (chitosan) (Ibrahim et al., 2019), negative (alginate), and neutrally (pullulan) charged polymers in blends. These blended samples were the starting point to further investigate the effect of a polysaccharide blend nano-emulsion for A375 drug delivery. Alginate, chitosan, and pullulan were each tested for their drug release profiles and the blended alginate-pullulan, pullulan-chitosan, and chitosan-alginate demonstrated improved release profiles. The optimisation procedure of the blended nano-emulsions led to the optimal nano-emulsion “pullulan-chitosan (NEPl2-Cs 1:2)”. The synthesized and folate-modified pullulan-chitosan nano-emulsion exhibited enhanced uptake in A375 cells. Furthermore, doxorubicin-loaded nano-emulsion substantially decreased melanoma viability.

As stated in section 3.2, nano-emulsion and drug combination acted successfully towards A375 melanoma cells causing cell death. Whilst the chemotherapeutic “doxorubicin” resulted in viable melanoma cells. This may be an indication of A375 resistance to doxorubicin and increased potential of drugs encapsulated within polysaccharide-based nano-emulsions to A375 melanoma cells (Capanema et al., 2018b). Considering initial bursts in release tests, induced cytotoxicity on keratinocytes/melanoma cell lines, and induced apoptosis capability, pullulan-chitosan nano-emulsion was chosen for further investigations. From the findings of the experimental work, polysaccharide-based nano-emulsions presented a promising potential for melanoma drug delivery. Alginate, chitosan, and pullulan were each tested for their effects on drug release. The blended alginate-pullulan, pullulan-chitosan, and chitosan-alginate samples demonstrated improved release profiles compared to non-blended nano-emulsions, whilst the optimisation procedure of the blended nano-emulsions led to the optimal nano-emulsion “pullulan-chitosan 1:2”.

Furthermore, doxorubicin-loaded nano-emulsion appears more efficient in delivering doxorubicin and inducing apoptosis in the melanoma cell line in this study. The current study and conclusion highlight the crucial impact of the deployment of suitable polymers and ratios in a drug delivery system as well as the importance of drug release patterns.

In conclusion, the developed formulation can be considered an important tool for the better uptake of drugs and in terms of melanoma drug resistance, further investigations are warranted. Furthermore, the optimal nano-emulsion can be considered as a potential delivery vehicle for other chemotherapeutics, and increased drug loading efficiency of nano-emulsion can be a promising tool for topical skin deliveries.

Declaration of Competing Interest

I do confirm that all the parties and involved researchers have been mentioned in this manuscript.

Data Availability

Data will be made available on request.

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