A novel low molecular weight nanocomposite hydrogel formulation for intra-tumoural delivery of anti-cancer drugs

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Herein, an injectable formulation composed of a low molecular weight gelator (LMWG) based hydrogel and drug-loaded polymeric nanocapsules (NCs) is described. The NCs, made of hyaluronic acid and polyglutamic acid and loaded with C14-Gemcitabine (GEM C14), showed a size of 40 and 80 nm and a encapsulation efficiency > 90%. These NCs exhibited a capacity to control the release of the encapsulated drug for more than 1 month. GEM C14-loaded NCs showed activity against various cancer cell lines *in vitro*; cell growth inhibition by 50% (GI_{50}) values of 15 ± 6, 10 ± 9, 13 ± 3 and 410 ± 463 nM were obtained in HCT 116, MIA PaCa-2, Panc-1 and Panc-1 GEM resistant cell lines respectively. Nanocomposite hydrogels were prepared using the LMWG - *N*4-octanoyl-2'-deoxycytidine and loaded for the first time with polymeric NCs. 2% and 4% w/v nanocapsule concentrations as compared to 8% w/v NC concentrations with 2 % and 3% w/v gelator concentrations gave mechanically stronger gels as determined by oscillatory rheology. Most importantly, the nanocomposite formulation reformed instantly into a gel after injection through a needle. Based on these properties, the nanocomposite gel formulation has potential for the intratumoural delivery of anticancer drugs.

[1] Abbreviations: low molecular weight gelator (LMWG); nanocapsules (NCs); *N*4-myristoyl gemcitabine (GEM C14); low molecular weight (LMW); hexadecyltrimethylammonium bromide (CTAB); Sodium hyaluronate (HA); poly-L-glutamic acid (PGA); GEM hydrochloride (GEM-HCl); 9-Diethylamino-5H-benzo[a]phenoxazine-5-one - Nile Red (NR); Linear visco-elastic (LVE) region; GEM resistant cell line (GEM R Panc-1).

1   Introduction

30   Supramolecular gels are formed from low molecular weight (LMW) molecules (typically < 3 kDa). The main characteristic of low molecular weight gelators (LMWG) is their ability to gel solvent (water in the case of hydrogels) at concentrations as low as 0.5% wt through the process of self-assembly and the formation of fiber networks (Sangeetha and Maitra, 2005). The self-assembly is governed by non-covalent physical interactions whose dynamic nature can bring two features: viscous flow under shear stress (shear-thinning)

35   and time-dependent recovery upon relaxation (self-healing) (K.J. Skilling et al., 2016). These properties make supramolecular gels appropriate for an injectable formulation.

The design of LMWGs for cancer therapy has been increasingly reported over the last decade. Specifically, peptide-type LMWGs have been extensively investigated and used for incorporation of various cancer drugs such as doxorubicin (DOX) (Naskar et al., 2009), campthothecin (Cheetham et al., 2013), paclitaxel

40   (Wang et al., 2011) and many others (Tian and Niu, 2014). Additionally, LMWG gel formulations, oligopeptides, lanreotide and degarelix composed of 8 and 10 amino acids respectively, are used clinically as hormone therapy in patients with neuroendocrine tumours (Pokuri et al., 2016; Steinberg 2009; Klotz et al., 2008; Vlieghe et al., 2010). Several LMWG formulations, specifically designed for intra-tumoural delivery of anticancer drugs have shown inhibition of tumour growth. For example, DOX was physically entrapped

45   in a gel made of L-alanine hydrazide gelator and was reported to reduce the tumour burden by 40% as compared to an untreated control when injected into a breast (4T1) tumour model in mice, whereas DOX alone had negligible antitumour effects upon injection (Singh et al., 2014). In another example, a Taxol derivative (succinic acid - glutathione) self-assembled into an injectable hydrogel and impeded tumour growth 2-fold compared to an i.v. injection of Taxol® in the breast (4T1) tumour model in mice. Furthermore,

50   the Taxol hydrogel was able to prevent metastasis. Additionally, the fatal dose of Taxol® in this hydrogel was shown to be at least 7.5x higher than that of Taxol® (Wang et al., 2012).

Based on the apparent success of gel formulations for the intratumoural delivery of anticancer drugs, the objective of this work was to design and develop a new nanocomposite hydrogel formulation consisting of a nucleoside based LMWG and polymeric coated nanocapsules (NCs). Considering that a variety of late

55   stage cancers cannot be surgically removed, (e.g. pancreatic and oesophageal cancers), it has been hypothesized that their treatment could benefit from such a drug delivery platform. The gel structure should ensure localised drug delivery over many cell cycles (Wolinsky et al., 2012) and the incorporation of NCs may provide opportunities for increased tumour permeation of drug, beyond that of diffusion from the gel (Strasser et al., 1995). Examples of such nanocomposite gels include: association of poly(ethylene glycol)-

60   block-poly(-lactic acid) (PEG-b-PLA) NPs with hydroxypropylmethylcellulose (HPMC) gels (Appel et al., 2015), Eudragit NCs (Contri et al., 2014), poly(d,l- lactide)-b-(ethyleneoxide) (PLA-b-PEO) NPs (Grant et al., 2005) and chitosan NPs (Şenyiğit et al., 2015) with chitosan gels, poly(ε-caprolactone)- poly(ethylene glycol)-poly(ε -caprolactone) (PCEC) NPs with Pluronic F127 gels (Gou et al., 2008) and some liposomal formulations with PEG gels (Lalloo et al., 2006). In comparison to polymer gels, LMWG are hypothesized

65   to have a different mechanism of release and pore structure and hence should be evaluated as a viable alternative to the extensively explored polymeric nanocomposite gels.

More precisely, in this work the association of *N*4-octanoyl-2'-deoxycytidine gels with polymer coated NCs loaded with *N*4-myristoyl gemcitabine (GEM C14), is presented as a delivery strategy of potential application for the treatment of non resectable tumours. A lipophilic (4-(*N*) position modification) form of

70   gemcitabine was selected to overcome the short half-life and acquired resistance of the parent compound (Mackey et al., 1998; Weizman et al., 2014; Pulido et al., 2014). Additionally, the hydrophobic modification of gemcitabine further allowed its encapsulation in the lipid core of NCs.

The selection of the drug nanocarrier to be include in the gel was based on our previous experience with polymeric NCs (Oyarzun-Ampuero et al., 2013; Rivera-Rodriguez et al., 2013; Lollo et al., 2015), (Janes et

75   al., 2001; Torrecilla et al., 2013),(Rivera-Rodriguez et al., 2013), (Gonzalez-Aramundiz et al., 2017) (PGA-PEG) (Borrajo et al., 2016). Hyaluronic acid (HA) and polyglutamic acid (PGA) nanocapsules were chosen as a good option based on previous studies showing their activity in vitro and in vivo (Orzayon-Ampuero et al., 2013; Lollo et al., 2015). Firstly these NCs were optimised for our application and their ability to inhibit cancer cell growth evaluated *in vitro* using cancer cell lines including a GEM-resistant pancreatic cell line

80   as drug resistance presents a major problem thwarting successful cancer treatment (Hong et al., 2009; Long et al., 2011). Finally, the formation and characterisation of a nanocomposite formulation, of NCs and a LMWG based hydrogel was assessed.

2   Methods

2.1   Materials

85   Miglyol® 812N oil - medium chain triglyceride (MCT) was kindly donated by CREMER OLEO GmbH & Co. Surfactants: Kolliphor® HS 15 (PEG-660-12 hydroxystearate) was kindly provided by BASF ChemTrade GmbH; Tween® 80- polyoxyethylene sorbitan monooleate and hexadecyltrimethylammonium bromide (CTAB) were purchased from Sigma. Polymers: Sodium hyaluronate 50 kDa (HA, LifeCore biomedical); poly-L-glutamic acid sodium salt 5 kDa - (PGA, Sigma). Drug GEM hydrochloride (GEM-HCl) was

90   purchased from Sigma. *N*4-myristoyl gemcitabine (GEM C14) and low molecular weight gelator: *N*4-octanoyl-2'-deoxycytidine were synthesised as previously reported (KJ. Skilling et al., 2018; K.J. Skilling et al., 2016). Fluorescent dye 9-diethylamino-5H-benzo[a]phenoxazine-5-one - (Nile Red) was purchased from MP Biomedical.

2.1.1   Reagents used for *in vitro* cell culture

95   Cell culture medium-RPMI 1640, phosphate buffer serum (PBS), foetal bovine serum (FBS, F7524); trypsin; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), methylene blue and the solvent DMSO were purchased from Sigma and used without further purification.

For the preparation of drug release dissolution medium, phosphate buffered saline (PBS) in the form of tablets were purchased from Sigma. One tablet was dissolved in 200 mL of deionised water yielding 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C.

2.2 Manufacture and characterization of nanocapsules

Polymeric NCs were produced by a self-emulsifying method that requires addition of an oil phase to aqueous phase under gentle agitation. Blending the oil, surfactants and drug (oil phase) in appropriate ratios at low heat (40-50 °C) until all were solubilised and then dispersing them directly in polymer solution (aqueous phase) at room temperature (RT) produces spontaneously oil-in-water NCs. The oil phase consisted of 70 or 53 mg of Miglyol® 812N; 45 or 53 mg of Kolliphor® HS 15; 45 or 53 mg of Tween® 80 and 0.25 mg of CTAB as surfactants per A or B formulation respectively. In addition, the oil phase contained (0.1% w/w) of drug GEM C14. The aqueous phase consisted of 2 mg/mL polymer solution of HA or PGA in pure deionised water (Milli-Q). The aqueous phase:oil phase ratio was 1:8 (w:w).

2.2.1 Physicochemical characterisation of NCs

The average diameter and polydispersity index (PDI) of NCs were characterised by dynamic light scattering after diluting the samples 3:100 (v:v) with Milli-Q water. Zeta potential (ZP) was determined by laser Doppler micro-electrophoresis after 5:100 (v:v) sample dilution with Milli-Q water. The measurements were performed on a Malvern ZetaNano ZS, UK.

Encapsulation efficiency was calculated as percentage of the drug in the isolated NCs compared to the total amount of the drug in NCs before the isolation. NCs were separated from the free drug using size exclusion chromatography columns (SEC) – Zetadex 25M, CentriPure P10. Columns were prepared according to the manufacture's protocol.

Colloidal stability of GEM C14 loaded NCs was assessed upon storage at room temperature (RT) or in the fridge (8 °C) for up to 4 weeks. In addition, colloidal stability was assessed upon incubation in PBS as well as in the cell culture medium (RPMI 1460) used in the *in vitro* cell culture studies. The dilution factor in these studies was 1:10 (v/v).

2.2.2 *In vitro* release of GEM C14 from NCs

The procedure for *in vitro* drug release involved incubation under sink condition of 0.5 mL GEM C14-loaded NCs (0.1 %) with 37.5 mL of release medium in a water bath at 37 °C with agitation at 100 rpm.. Other volumes of release medium were employed, 12.5, 24.5 and 37.5 mL, where the latter two showed negligible effects on the released amount, hence further dilutions were not explored. At predetermined time intervals 1 mL of sample was taken and the separation of the free drug from the NCs was performed by size exclusion chromatography (SEC). The sample was replaced with 1 mL of PBS. The percentage cumulative release was calculated indirectly using the following equation:

$$\% \ cumulative \ release = \frac{Xi - Xt}{Xi} \times 100$$

where Xi is the amount initially loaded into NCs and Xt the amount that is in NCs at time point. As drug released was below the detection limit, by measuring the drug retained within NCs, the amount of drug released could be indirectly calculated. The small amount discarded at each time point when removing sample and replacing it with PBS was added to the amount released.

Quantification of GEM C14 by HPLC was carried out using a method based on those already reported in the literature (Bastiancich et al., 2016; Immordino et al., 2004). Briefly, GEM C14 and GEM C14 loaded NCs were dissolved in methanol and an aliquot was injected into a HPLC system consisting of an ACE 5 C18 5 μm; 75 x 4.6 mm column followed by isocratic elution (80% methanol, 20% pure water) at 30 °C, under constant flow rate of 1 mL/min and UV detection at 248 nm. These conditions gave a retention time of around 7.8 minutes. The standard calibration curve of GEM C14 was linear ($R2 > 0.999$) in the range of concentrations between and 0.5 and 50 μg/mL.

2.2.3 *In vitro* activity of drug-loaded NCs in cancer cells

GEM C14-loaded NCs were manufactured as described above and were assessed for *in vitro* cell growth inhibition on cancer cell lines. Eight formulations (HA 80, HA 40, PGA 80 and PGA 40 nm NCs blank or loaded with GEM C14) were tested and compared with GEM C14 and GEM hydrochloride (GEM-HCl). The initial concentration of GEM C14 in NCs was ~ 25 μM. Blank NCs did not contain drug. Human carcinoma cell lines used in experiments were the HCT 116 colorectal and MIA Paca-2, Panc-1 and GEM-resistant Panc-1 pancreatic cancer cell lines. Human carcinoma cell lines (HCT 116, MIA Paca-2 and Panc-1) were all sourced from the American Type Culture Collection (ATCC).

General cell culture involved incubation of serially sub-cultured cells at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cells were grown in RPMI 1640 medium supplemented with 10% v/v FBS. Once cells had reached approximately 70% confluence they were used for the experiments. A pancreatic GEM-resistant cell line was generated by continuous culture of parental Panc-1 cells in medium supplemented with incremental gemcitabine concentrations, and then maintained in medium spiked with 1 μM Gemcitabine. This cell line was developed and kindly donated by Dr Lodewijk Dekker (University of Nottingham).

2.2.3.1 *Growth inhibition assay*

The MTT assay was used for mitochondrial activity evaluation, a surrogate marker for viable cell number. Briefly, cells were seeded into 96-well plates at a density of 3 x10^3 cells/well in 180 μL of culture medium. An extra plate was prepared containing cells from each cell line in order to carry out an MTT assay at the time the cells were treated. This allows the initial optical density (OD) to be determined. After 24 h, cells were treated with drug alone (GEM C14 and GEM-HCl), blank NCs and GEM C14-loaded NCs starting with

GEM concentration from 25 μM followed by 10-fold serial dilutions. Four wells were used for each condition. Control cells were treated with medium alone. Following 72 h incubation, 50 μL of 2 mg/mL MTT solution were added to each well. Cells were incubated for a further 2.5 h to allow formazan production. The aqueous medium was then aspirated and the formazan product solubilised in DMSO (150 μL/well). Absorbance at 570 nm (optical density - OD_{570}) was read using an EnVision 2104 Multilabel Reader. The concentrations at which cell growth is inhibited by 50% (GI_{50}) were calculated from the dose-response curves estimated by linear interpolation between two closest values after taking into account the initial optical density acquired at the time of treatment. MTT assays were carried out on ≥ 3 separate occasions and mean GI_{50} values were calculated.

2.2.4 Gel-NC composites for localised cancer therapy

*N*4-octanoyl-2'-deoxycytidine was used as LMWG as it was reported to gel in pure water using a heating-cooling cycle as the method for gel formation (Skilling et al., 2016). A defined amount of gelator was added to water and heated in a water bath up to 60 °C until the compound completely dissolved. The sample was then allowed to cool to room temperature for ~ 30 minutes. For the preparation of gel-NCs composite, the procedure was the same except that the gelator was dissolved in NCs formulation instead of in pure water. The following concentrations of LMWG were investigated: 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4% (w/v). All 4 NCs` formulations were tested (HA 80, HA 40, PGA 80 and PGA 40 nm NCs). HA 80 nm NCs were tested at concentrations of 8; 4 and 2% w/v. For the comparison among different NCs, all NCs` formulations at a concentration of 4% w/v were formulated with a 2% w/v gelator concentration.

2.2.4.1 Measurement of storage and loss modulus

Oscillatory rheology was carried out using an Anton Paar MCR302 Modular Compact Rheometer using a cup and vane geometry. Gel sample (2 mL) was placed in a disposable measuring Cup EMB-Z4 (Anton Paar) which was set into the lower part of the rheometer (cup). The upper part of the rheometer (vane) was positioned into the centre of the gel at a depth of 2 mm. All gel samples were prepared 1 day before the rheological measurement. Oscillatory amplitude sweep measurements were carried out at strains varying between 0.05 – 100 %. The G'and G" (storage modulus and loss modulus, respectively) were measured at a constant angular frequency of 10 rad/s. The temperature was set to 37 °C to mimic physiological conditions. The linear visco-elastic (LVE) region was calculated using Rheoplus Anton Paar software where deviation from linearity was < 3-6%.

2.2.4.2 Release of the NCs from the gel-NCs composite

Nile Red (NR) loaded NCs were used in the *in vitro* release experiments of NCs from the gel nanocomposite formulation. The preparation of NR-loaded NCs involved addition of ethanol solution containing NR into the oil phase. After blending all components together, the oil phase plus dye, was stirred for several hours at 80 °C to ensure that all ethanol had evaporated. NCs were formulated, as previously described by dispersing oil phase into polymer solution under magnetic stirring. The final theoretical NR concentration in

NCs was ~ 10 µg/mL. The size of NCs loaded with dye was determined using the Zeta sizer as previously described.

Release of NR-loaded NCs from the gel system was performed in a similar way as from NCs. The gelator (2% w/v) was formulated with 8% w/v HA 80 nm NCs; 4% w/v HA 80 nm NCs or 4% w/v HA 40 nm NCs loaded with NR. Gels were formulated with 0.5 mL of NCs 1 day before the experiment and then topped up with 12.5 mL of release medium (PBS, pH 7.4). Samples were stored at 37 °C in a water bath. At predetermined time points, a sample was taken from medium above the gel, the amount of NR released from the gel was quantified and cumulative release was calculated. At this point, there was no separation step as only a low percentage of NR was released (i.e. 20% released after 72 h). For the NR quantification, aliquots of release samples were diluted with PBS and analysed using a plate reader SPARK® 10M, Tecan at 553/636 nm excitation/emission wavelengths.

2.2.4.3   *The evaluation of NC integrity*

The process of gel formation involved gelator solubilisation at elevated temperature which can impact NC properties, thus their integrity was assessed. This was done by heating, both, NCs dispersion and gel-NC composite to 60 °C. At this temperature, the gelator is expected to dissolve, liberating the NCs. A sample was collected before the gel restructured, and NC size was determined as described above (2.2.1).

2.2.4.4   *Injectability of the gel nanocomposite*

Evaluation of gel reformation after injection involved dissolution of the gelator in NR HA 80 nm NCs at 60 °C. While still in liquid form, the formulation was then loaded into a syringe and allowed to cool to RT (~ 30 min). When the gel had formed, the syringe contents were dispensed through a needle (25 G) into a vial.

3   Results and discussion

The objective of this work was to develop a novel injectable composite intended for intratumoural delivery of anti-cancer drugs. The composite consist of HA NCs dispersed in a LMWG. The production and characterization of the NCs, the gel and the resulting composite is discussed as follows.

3.1 Production of HA and PGA NCs

HA or PGA NCs were produced by self-emulsification, without the use of any solvents. Considering the first objective of this work, to reduce their size below 100 nm, the influence of the amount of oil (Miglyol® 812N), and the amount of surfactants (Tween® 80 and Kolliphor® HS 15) was investigated as described in the supplementary information (ESI-S1). The excipients and physico-chemical characteristics of HA and PGA NCs of two sizes (i.e. 40 and 80 nm) and loaded with GEM-C14 are given in Table 1. All formulations exhibited low polydispersity (PDI ≤ 0.2). The charge of the NCs depended on the polymer coating with zeta potential values closer to neutral when PGA was used (-10 to -4 mV) while more negative charge was observed for HA NCs (-19 to -9 mV). Our lab has previously reported the preparation of polymeric NCs

(Protamine) with a size close to 200 nm, using a self-emulsifying technique (Jakubiak et al., 2017), however, here we describe for the first time the production of HA and PGA NCs below 100 nm using a self-emulsification process. The formation of nanoemulsions with a globule size <100 nm, first at elevated temperatures (85 °C) (Heurtault et al., 2003) and later at 30 °C has also been reported (Lefebvre et al., 2017; Saberi et al., 2013). However, these emulsions did not have a polymer shell as those reported in this work.

A high encapsulation efficiency (> 90%) of GEM C14 is obtained in all formulations indicating compatibility between the drug and the core of NCs. High encapsulation efficiency (> 70%) of lipophilic compounds is a characteristic for NCs (Mora-Huertas et al., 2010) and our report is in accordance with other self-emulsifying NCs (Jakubiak et al., 2017).The size and spherical shape of the NCs were additionally confirmed by TEM imaging (see ESI –Figure S2).

The drug loading (0.1 w/w) is sufficient for intra-tumoural delivery as will be illustrated by comparison to the closest literature example of Lee et al. These authors demonstrated that a polymeric film designed for treatment of gastrointestinal cancer contained 150 μg of GEM per implant and showed appropriate loading as it induced complete regression of CT-26 colon carcinoma s.c. model in mice (Lee et al., 2012). With a drug loading of 0.1% (w/w) or 80 μg/mL of GEM C14 (assuming 8% w/w oil/surfactant in the nanoemulsion) we can achieve 16 μg per tumour (max volume that can be injected is typically 200 μl). This drug loading could potentially be sufficient for GEM C14 as compared to GEM as the lipophilic derivative will have increased retention at the administration site and also has nanomolar potency as demonstrated below (see Figure 3).

Table 1 Excipients and Physico-chemical characteristics of different NCs loaded with GEM C14 (0.1% w/w). Mean values (n = 3) are shown, error bars represent standard deviation of the mean, where n is number of independent trials. All NC formulations contained 0.25 mg CTAB The aqueous phase:oil phase ratio was 1:8 (w:w). All amounts are expressed in mg per formulation, where final formulation volume is 1.5 ml.
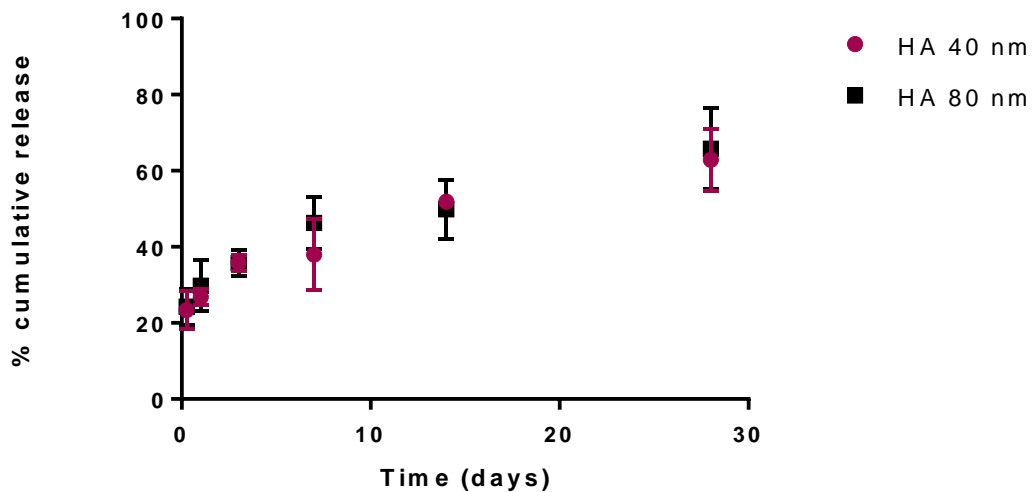***HA 80 nm NCs*** *represent hyaluronic acid coated NCs with a diameter of 80 nm; whilst **PGA 80 nm NCs** are poly-L-glutamic acid coated NCs with a diameter of 80 nm; **HA 40 nm NCs** are hyaluronic acid coated NCs with a diameter 40 nm; and **PGA 40 nm NCs** are poly-L-glutamic acid coated NCs with a diameter of 40 nm.*

Formulation name	*NC Excipients*				*NC characterisation*			
	Kolliphor® HS 15 (mg)	**Tween® 80 (mg)**	**Miglyol® 812N (mg)**	**HA or PGA (mg/ml)**	**Size (nm)**	**PDI**	**ZP (mV)**	**EE (%)**
HA 80 nm NCs	45	45	70	2	79 ± 6	0.2	-19 ± 1	95 ± 8
PGA 80 nm NCs	45	45	70	2	79 ± 5	0.2	-10 ± 1	90 ± 5
HA 40 nm NCs	53	53	53	2	40 ± 2	0.2	-9 ± 1	92 ± 7
PGA 40 nm NCs	53	53	53	2	34 ± 2	0.1	-4 ± 1	90 ± 5

The importance of the NCs formulations developed herein is reflected by the known influence of the physicochemical properties of nanocarriers on their diffusion through the tumour stroma (Gratton et al., 2008; Ernsting et al., 2013). (Lee et al., 2010; Jiang et al., 2008). Generally, smaller particles i.e. ≤ 100 nm perform better in terms of accumulation and distribution in tumours (Chauhan et al., 2012; Saw et al., 2018; Cabral et al., 2011; Meerovich et al., 2008). Moreover, in a recent publication by our group, PGA-PEG NCs of 100 nm were found to be drained into lymph nodes faster than the 200 nm NCs of the same composition (Abellan-Pose et al., 2016). Although the majority of the work in the literature describes the intravenous administration of nanoparticles, intratumoural injection (the intended application of our nanocomposite hydrogel) of small NCs locally also has the potential to give increased penetration through the tumour as described for Pegylated gold nanoparticles (50 nm), an emulsion (85 nm) and neutral liposomes (120 nm). Whilst an 250 nm emulsion and 100 nm liposomes with positive surface charge had limited diffusion (Laprise-Pelletier et al., 2018; Nomura et al., 1998).

3.1.1   *In vitro* release of GEM C14 from NCs

We progressed to the release studies of GEM C14 without undertaking drug stability studies as very similar alkanoyl gemcitabine derivatives were previously established as being stable *in vitro* (Immordino et al., 2004) . In addition, for other N-alkanoyl gemcitabine derivatives the amide linkage was also demonstrated to be stable between 6 and 8 in vitro (Wickremsinhe et al., 2013; Bender et al., 2009). The release studies were carried out under sink conditions, by incubating the NCs in PBS at 37 °C for 1 month. The amount released from HA 40 and HA 80 nm was evaluated   by analysis of the amount remaining in the NCs and then subtracting this from the initial amount encapsulated. Both formulations showed a similar biphasic drug release profile (Fig. 1), characterised by an initial burst release of ~ 20% followed by a second phase in which further ~ 40% of drug was released over 1 month. The initial burst release maybe attributed to the non-ionic surfactants solubilising drug at the oil water interface (Youm et al., 2014). The total amount of GEM C14 released from the NCs after one month was 63 ± 8 and 66 ± 10 % from HA 40 and HA 80 nm NCs respectively. The amount of drug that remained encapsulated in NCs during the release studies is notable (~ 35% after 1 month) thus showing its stability inside the nanocarrier and the ability of the nanocarrier to maintain sustained release. Similarly, Bastiancich *et al.* reported 56% of lauroyl-gemcitabine (GEM C12) released in the first 48 h and 77% released after 1 month (Bastiancich et al., 2016). Delayed release of GEM C14 (with a longer carbon chain compared to C12) can be attributed to its higher affinity towards the oil core and the diffusion between the hydrophobic core and surrounding environment can be hindered by the packing density of the surfactant molecules and the presence of the polymer coating (Lamprecht et al., 2002; Poletto et al., 2008). These data suggest the potential for sustained release in future *in vivo* studies.
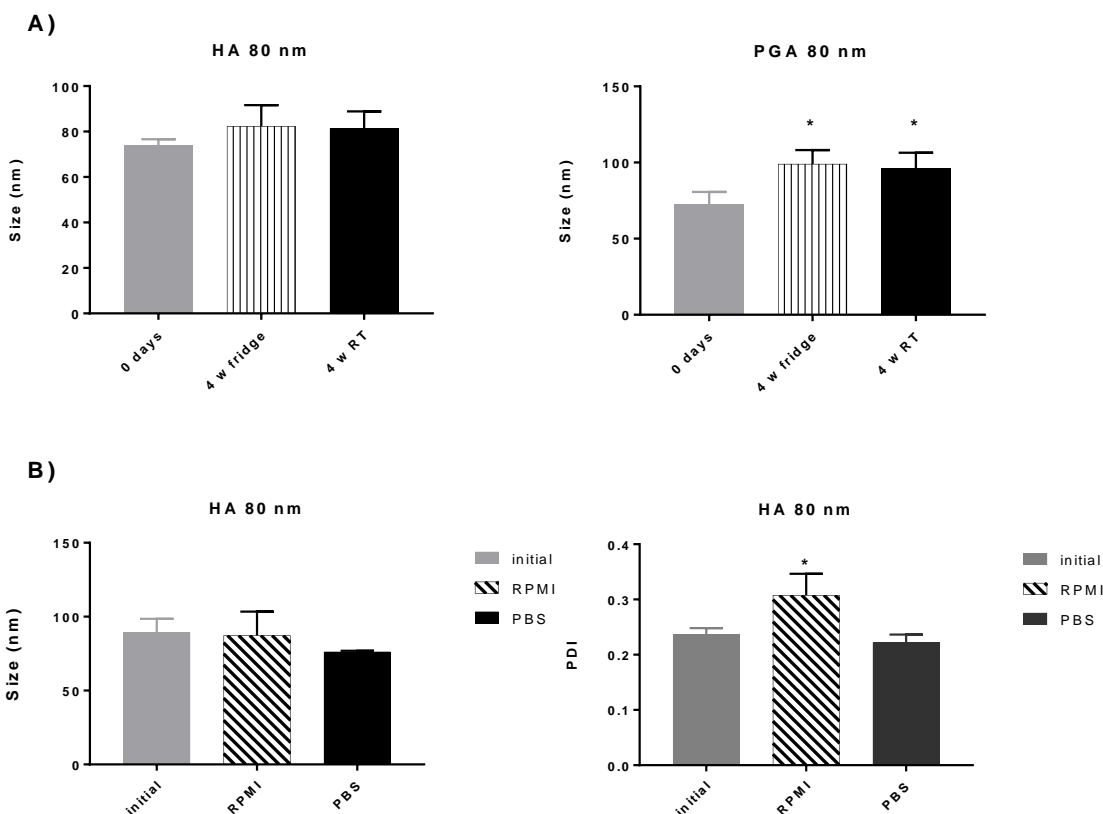
3.1.2   Stability of NCs upon storage and after incubation in bio-relevant media

From an industrial and translational perspective, it is important to obtain formulations that are stable upon
300   storage. Additionally, a critical aspect of physiological relevance is their stability in bio-relevant media as it
is known that stability in biological media can be a barrier in the development of many nanoformulations
(Moore et al., 2015). Therefore, storage stability at RT and in the fridge as well as the stability of the NCs
in PBS, pH = 7.4 and in RPMI cell culture medium was investigated.

In the stability study, neither aggregation nor precipitation of nanoparticles were observed during storage ≤
305   1 month. This good colloidal stability profile, which could be attributed to the electrostatic repulsion between
the negatively charged NCs agrees with the stability data reported for other polymeric based NCs (Lozano
et al., 2013; González-Aramundiz et al., 2015; Zanotto-Filho et al., 2013; Abellan-Pose et al., 2016).
Although, changes in particle size of the nanoparticles throughout the entire course of the study were
minimal, PGA NCs exhibited significant (1-way ANOVA Dunnett's multiple comparison test, $p < 0.05$)
310   increase in size after 4 weeks storage for both PGA 40 and 80 nm NCs. This could be the result of film
thinning and NCs coalescence due to the lower polymer coverage (assessed by ZP) which led to weak
repulsive forces between PGA NCs compared to HA NCs.

The size of HA 80 nm NCs did not change significantly after incubation in PBS nor in RPMI. However, a slight increase in the PDI value ($p < 0.05$, Dunnett's multiple comparison test) was observed upon incubation of the NCs in RPMI medium. This could be a consequence of higher salt content (2x potassium ions) in the RPMI medium and is more likely due to the potential interaction of the proteins from the FBS serum in the RPMI, with the NCs. The presence of polymers and other molecules on the surface of particles is a well-known approach for conferring electrostatic and steric stabilisation to NCs (Tan et al., 2010; Storm et al., 1995; Moore et al., 2015).

3.1.3 *In vitro* activity of GEM C14 loaded polymeric NCs in cancer cells

Human colorectal and pancreatic carcinoma cells were exposed to serial dilutions of NCs (blank or loaded with the drug) and to drug alone for 72 h. Subsequently, MTT assays were carried out to assess cell viability and GI_{50} values are presented in Figure 3. GEM C14-loaded NCs as well as free drugs (GEM-HCl and GEM C14) gave nanomolar GI_{50} values in all wild type cancer cell lines (HCT 116, MIA PaCa-2 and Panc-1), demonstrating high potency. On the other hand, blank NCs (no drug) exhibited a 10 to 100-fold higher

GI$_{50}$ values than drug containing NCs implying that growth inhibition of these cancer cells is mainly due to the drugs activity. Representative dose response curves are shown in ESI-Figure S6 for HCT 116 and MIA
335　PaCa-2 cell lines and Figure S7 for Panc-1 and GEM resistant Panc-1 cell lines.
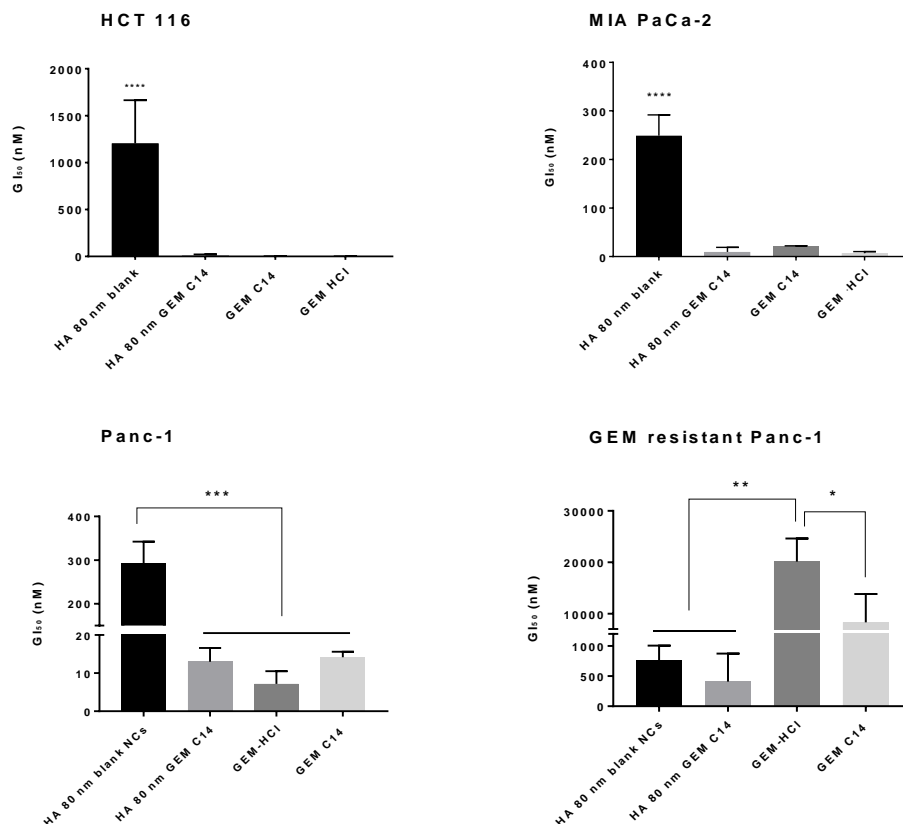
Figure 3 Mean GI$_{50}$ values (nM) ± s.d. in HCT 116, MIA PaCa-2, Panc-1 and GEM resistant Panc-1 cell lines following 72 h treatment, with HA 80 nm NCs blank or loaded with GEM C14 and for naked drugs: GEM-HCl or GEM C14. One-way ANOVA multiple comparisons test ****(p<0.0001); ***(p<0.001); **(p<0.01); *(p<0.05). Mean values are shown;
340　*error bars represent standard deviations of 3 independent trials.*

A pancreatic GEM-resistant cell line (GEM R Panc-1) was obtained by continuous culture of parental Panc-1 cells in gemcitabine and maintained with gemcitabine at a dose of 1 μM. In this cell line, free drug in the form of either GEM C14 or GEM-HCl exhibited negligible growth inhibitory effects with GI$_{50}$ values in the μM range confirming its resistance towards not only commercial GEM-HCl but also towards the modified
345　lipophilic GEM C14. Activity of GEM C14 loaded NCs in GEM resistant Panc-1 cell line showed 20 to 50 times lower GI$_{50}$ values compared to those of GEM C14 and GEM-HCl respectively. Interestingly, in contrast to data on wild type cancer cell lines, blank NCs in GEM resistant Panc-1 cell line showed similar activity to drug loaded NCs. For this drug resistant cell line, growth inhibition appears to be a consequence of the NC formulation which includes both non-ionic surfactants and a small amount of a cationic surfactant i.e
350　CTAB. CTAB has been described as a cytotoxic agent (He et al., 2011; Ito et al., 2009). The presence of

CTAB in blank NCs may possibly explain the similar toxicity of empty and loaded NCs on the GEM R pancreatic cell line.

3.2 Association of polymeric NCs and LMWGs

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*N*4-octanoyl-2'-deoxycytidine was chosen as a gelator on account of the absence of solvents used in its formulation, thus its compatibility with NCs. Initially, hydrogels consisting of only gelator and water were rapidly formed after a heating-cooling cycle. The storage modulus (G') derived from the LVE region of amplitude sweep tests was used as a measure of gel strength (Fig. S3). Results indicated that the concentration of gelator can influence gel strength in the hydrogel only and in nanocomposite hydrogels with NCs (Figure 4). For pure hydrogels, G' values increased from 148 Pa to 1760 Pa in gelator concentration range between 0.5 and 2% w/v. However, for the highest concentration (2.5% w/v), the value of the elastic modulus dropped to 517 Pa. The influence of the gelator concentration on the value of the elastic modulus has been reported elsewhere (Friggeri et al., 2004; Frith et al., 2015). With increases in gelator concentration the elastic modulus will increase until it reaches a plateau (Mohmeyer and Schmidt, 2005) or a decrease in G' values can be observed above certain concentrations. At higher concentrations the gelation process can almost be instantaneous resulting in shorter fibers and consequently reduced elasticity of the system or can lead to precipitation and the disruption of the gelator network (Menger and Caran, 2000). In the case of gel-NCs composite, it is important to note that the gelator, *N*4-octanoyl-2'-deoxycytidine, at a concentration of 0.5% w/v failed to gel in the presence of NCs. Whereas with 1% w/v gelator concentration a gel was macroscopically formed but with a short LVE region. However, further increases in gelator concentration improved the linearity of the LVE region and resulted in increased gel strength (Fig. S3 B). When pure hydrogel and gel-NCs composite are compared at the same gelator concentration (2.5% w/v) a 10-fold increase in G' values in the presence of NCs is noted. Additionally, gel strength was independent of NCs' size (40 or 80 nm) or polymeric coating (hyaluronic acid or poly-glutamic acid) as their G' were not significantly different (ESI-S4).

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As we have seen, the presence of NCs limits the ability of gel formation below certain gelator concentrations which implies that the amount of NCs can also play an important role in optimal LMWG-NCs composite formulation. Figure 4 shows that a higher amount of NCs (8 vs 2 and 4%) decreases the elastic modulus of the nanocomposite gel formulations regardless of the gelator concentration. Unlike polymeric gels, the *N*4-octanoyl-2'-deoxycytidine LMW gelator is a small molecule which in the presence of polymeric NCs hypothetically can participate in oil-water interface of lipid NCs due to its amphiphilic structure. As the amount of NCs increases, the number of gelator molecules at the oil/water interface can also potentially increase and subsequently decrease the gelator involvement in the formation of gel network resulting in the formation of a gel with a weaker structure. These data are of high importance as it is the first time that nanocomposite formulation of a LMWG and polymeric NCs has been evaluated; only composites of LMWG and inorganic and metal particles have been previously reported (Ghosh et al., 2017; Mitra and Das, 2008; Nanda et al., 2012; Song et al., 2010). Recently Ghosh *et al.* have showed that a hydrogel comprised of

hydroxyapatite (HAP), fluorenyl-9-methoxycarbonyl diphenylalanine (FmocFF) and Fmoc-arginine (which does not gel but mediates high affinity to HAP) gave novel hydrogels with mechanical strength greater than the gel alone appropriate for bone regeneration ie. 29 KPa. Whereas decreases in elastic moduli were reported by (Nanda et al., 2012), where thiol (-SH) containing amino acid/peptide ligands have been used to stabilize small silver nanoparticles, and then they were incorporated into a N-terminally Boc (tert-butyloxycarbonyl) group-protected tripeptide (Boc-Phe-Phe-Ala−OH). In this paper the concentration the nanoparticles was an important parameter and their proposed model was that the interaction of the functional group on the surface interferes with gelator self-assembly and weakens the gel. However in the nancomposite gel our proposed model is that there is hydrogen bonding between the nanocapsule polymer shell and the pentose sugar exposed on the external surface of fiber (as demonstrated in a previous paper from our group (Angelerou et al., 2018)) that binds the nanofibers together.
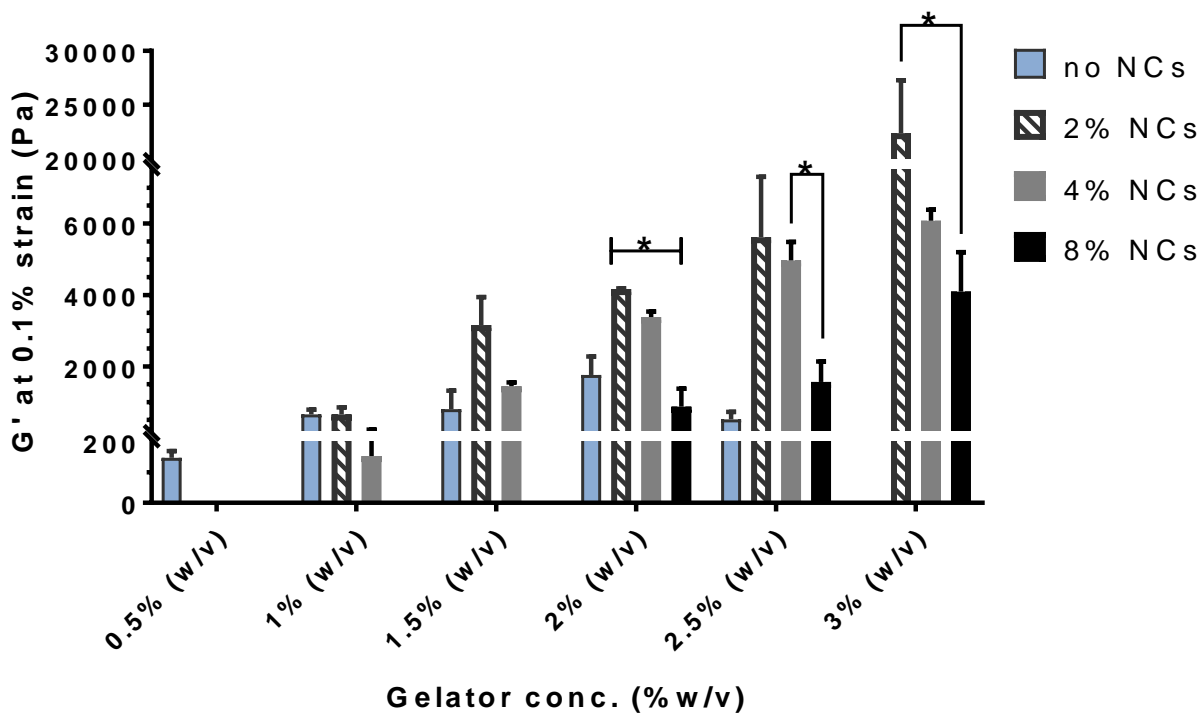
Figure 4 Influence of concentration of NCs (2, 4 and 8% w/v) on gel strength (G') in gel-HA 80 nm NCs systems. The effect is compared among various gelator concentrations: 1, 1.5, 2, 2.5, 3% w/v. Tests were performed a day after gel preparation at 37 °C, γ= 0.05 – 100% and ω=10 rad/s). Mean values are shown; error bars represent standard deviations of 3 independent trials. 1 way ANOVA Tukey's multiple comparisons test with *(p<0.05).

3.2.1 *In vitro* controlled release of NCs from the gel

Finally release of NCs from gel composite was assessed. For this experiment, NR was loaded into the NCs as this hydrophobic compound has high affinity towards the oil core as shown in the ESI-Figure S8 with ~80% retained in the NC after 72 h . Initially a ratio of gel to release medium of 1:5 (v:v) was chosen after

410   reviewing release conditions from NCs and gel formulations (Appel et al., 2015; Bastiancich et al., 2016; Bunzen and Kolehmainen, 2013). However, this volume of buffer was found to be too low for the release assessment from gels, since the whole release medium gelled after 24 h. This can be explained by gel disassembly in contact with water and reassembly of fibers once the gelator was distributed in the medium. Therefore, 12.5 mL was added to 2% w/v gel prepared in 0.5 mL NCs and no gelling of the medium

415   occurred. Hence these conditions were used for further release experiments. Although, different sizes of NCs and different amounts of NCs were compared (Figure 5) release plots show similar profiles. The gel containing 8% NCs released NR slightly quicker than the gel with 4% NCs. These data are in accordance with gel strength. Nanocomposite gels formed with fewer NCs (4%) demonstrated higher G' values and retained NCs longer in the composite, compared to formulation containing 8% NCs. Although not significant,

420   gels containing 80 nm NCs showed faster release of NR-loaded NCs than gels containing smaller 40 nm NCs. Similar observations were reported for lipid NCs where GEM C12 plays the role of gelator where NCs of 29 and 55 nm showed negligible difference (10-20%) in release from their gel (Moysan et al., 2014). % released decreases at 72 h as there is some partitioning of the NR into the release medium.
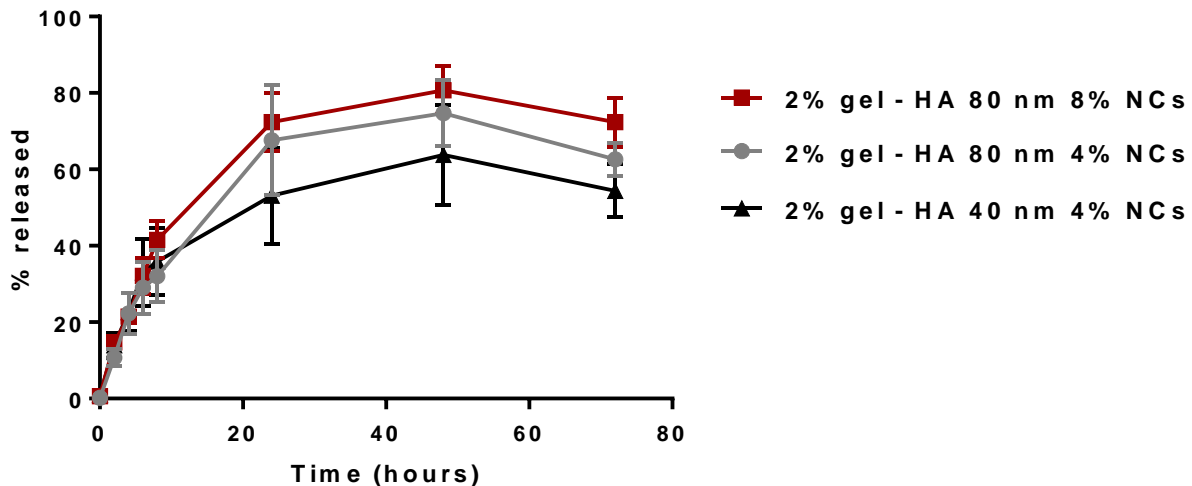
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Figure 5 Cumulative release of NR-loaded NCs from gel-NCs formulation at 37°C in PBS medium, pH=7.4. Concentration of gelator in all systems is 2% w/v and NCs used in this experiment are: HA 80 nm NCs of 4 and 8% w/v

Following the release of NCs from the gel, the size of NCs released in the medium was examined. Released NCs of 172 ± 24 nm were observed, which is larger than the initial size of NCs (~80 nm). Hence the integrity of NCs was evaluated under conditions of gel preparation. In Figure 6, the size of NCs in the presence or

435 absence of gelator after heating at 60 °C is presented. NCs heated to 60 °C exhibited only a slight increase in size to 55 ± 3 and 98 ± 17 nm for HA 40 and HA 80 nm formulations respectively. However, in the presence of gelator, the size of NC for both systems, increased up to ~ 200 nm. It seems that it is not the temperature that causes increase in size of NCs, but the presence of gelator around NCs. PDI values for all samples were ≤ 0.2 suggesting that no aggregation occurred. How much this increase in size would limit

440 the diffusion in the tumour mass is difficult to predict. There is limited data in the literature describing the diffusion of nanoparticles across the tumour. These data indicate that nanoparticles with a size of 250 nm or a positive surface charge may have a limited diffusion(Laprise-Pelletier et al., 2018; Nomura et al., 1998). According to this, the NCs described in the manuscript are expected to have adequate diffusion across the tumour.

445 Irrespective of the size observed by light scattering, using TEM imaging it was possible to confirm the presence of NCs < 100 nm diameter, dispersed in the gel (Figure 6B). The presence of fibrous network is clearly seen with homogenous distribution of spheroid particles in between fibers. Therefore, it could be inferred that the increase in size observed by light scattering is not due to the aggregation of the NCs but rather associated to the presence of fibers around the NCs.
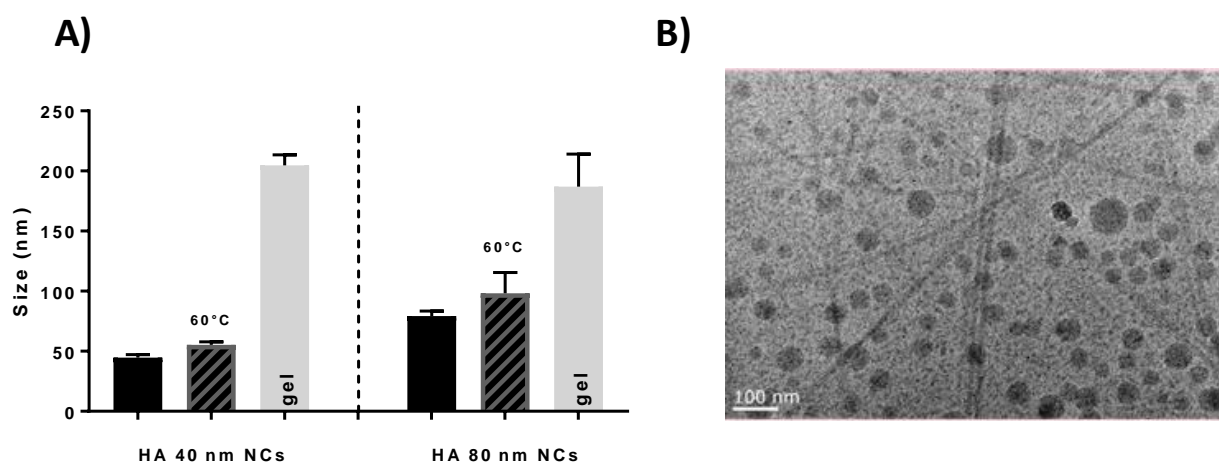
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Figure 6 A) Change of initial size of HA 40 or HA 80 nm NCs after being exposed to heat at 60-70 ˚C with and without gelator. Black bars present size of NCs alone before the heating, black bars with grey lines NCs alone after the heating

3.2.2   Injectability

When considering a formulation for direct intra-tumoural delivery, injectability of the system is a crucial characteristic to evaluate. Our group has previously reported 70% gel recovery, after a time-dependent rheology test, for the *N*4-octanoyl-2'-deoxycytidine. In Figure 7, an image of a syringe loaded with gel-NCs composite formulation, and the appearance after its contents were dispensed through the needle is shown. The gel formulation with NCs reformed instantly and remained in its gel form. The vial has been inverted to show that the gel has retained its viscoelastic properties after passing through the needle. Furthermore, their ability to be placed in tissue was tested in an *in vitro* simulation of intra-tumoural delivery i.e. chicken thigh. To mimic a resection site and intra-tumoural delivery, we bored out a hole in chicken thigh and used a syringe (without needle) to place the gel inside and observed the shape of the gel. Formulations with the same gelator concentration (2% w/v) and different amounts of NCs (8 and 4% w/v) were compared in terms of injectability. While the gel containing 4% w/v NCs retained its structure, the gel containing 8% w/v NCs did not reform after dispensing through a syringe (images in ES1- S5).
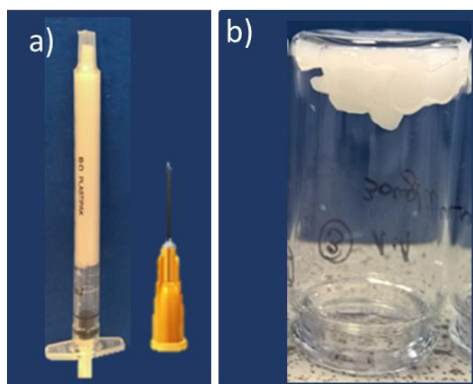

Figure 7 Gel formulation (3% w/v gel- 4% HA 80 nm NCs systems) loaded in a syringe (a) and dispensed through the syringe using needle (25G) into the vial (b).

Several LMWG formulations and gel-nanoparticle composites (Chaudhari et al., 2015; Appel et al., 2012) have shown the ability to recover near-total initial strength. We have observed here that gel structure is not compromised after injection of the nanocomposite hydrogel formulation and this is of significant importance for future *in vivo* experiments.

4   Conclusions

A novel nanocomposite formulation, consisting of an in-situ forming hydrogel containing polymeric NCs, has been developed. The hydrogel, here described for the first time, is made of a low molecular weight gelator (N4-octanoyl-2'-deoxycytidine) and has been shown able to accommodate a significant amount of NCs within its fibrillar structure. The NCs, produced by a self-emulsification technique, were a very small

size (40-150 nm) and provided a controlled release of the drug encapsulated for more than one month. Specific features of this nanocomposite formulation include (i) adequate mechanical strength; (ii) capacity to control the released of the nanocapsules; and (iii) ability to gel *in situ* upon injection. This formulation could have potential for intratumoural delivery of anticancer drugs in the case of unresectable cancers such as gastric, pancreatic or oesophageal cancer. This delivery technology could also be of interest as a long acting injectable for subcutaneous or intra-cavital administration.

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