UTILIZATION OF NOVEL SELF-NANOEMULSIFYING FORMULATIONS (SNEFs) LOADED PACLITAXEL FOR THE TREATMENT PROSPERITY OF BLADDER CANCER

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Abstract

Background*:* Limited drug penetration into solid tumors has been one of the potential causes of resistance to chemotherapy for the treatment of bladder cancer. The aim of the study is to develop non-toxic self-nanoemulsifying formulations (SNEFs) of paclitaxel and to evaluate their ability to serve as a tool increasing the stability and solubility of paclitaxel in formulations utilizing the drug intravesical administration. ***Methods****:* Various oil-in-water non-toxic self-nanoemulsifying formulations (SNEFs) were developed using Cremercoor MCT, Kollisolv MCT, Miglyol 812, 810, Capmul MCM, Imwitor 988, Imwitor 742 with TO106V, Tween 85, HCO30, Kolliphor EL and Cremophor RH40 at size ranges from approximately 19 to 110 nm that are capable of enhancing the solubility and stability of paclitaxel. Visual assessment and droplet size measurements were taken into initial consideration for optimised SNEFs. Paclitaxel was added with the oil/surfactant mixture before dispersing the mixture in water to form SNEF. The cytotoxicity of the optimal SNEFs was compared with the raw paclitaxel dispersion *in vitro*. ***Results***: Initial characterisation and solubility studies showed that mixed glycerides of Kollisolv MCT/Imwitor 742 with water-soluble surfactant (high HLB) containing formulations generated highly efficient SNEFs as they are stable and produced lower nanodroplets with higher drug loading. The results have demonstrated that the SNEFs have good ability to retain its characteristics under conditions similar to that found in the urinary bladder up to 48 hours. However, the results also showed that chemosensitivity of cancer cells exposed to paclitaxel was attenuated in the presence of SNEFs. Larger size SNEFs have shown to induce more inhibitory effects on paclitaxel activity. ***Conclusion***: optimised SNEFs have demonstrated the ability to enhance the solubility of paclitaxel with stable construction of SNEFs under variable physiological conditions. The reduction of the efficacy of SNEF-loaded paclitaxel indicates a strong

59 encapsulation of the drug within the nano-carriers causing limitation in drug release.
60 Such drug encapsulation may facilitate the penetration due to reduced cellular
61 metabolism the drug. Further investigations are warranted.
62

1. Introduction

Tumor resistance to chemotherapy has been the major problem facing cancer therapy for all types of cancers. The failure of chemotherapy to reach and kill all cancerous cells will lead to probable tumor recurrence and possible progression of cancer to muscle-invasive tumor [1, 2].

Bladder cancer, which is considered one of the main types of cancer, is known for its high rate of recurrence in patients after a short period of eradication. Despite the advances achieved in bladder cancer treatment and decreased mortality rates during the last two decades, yet a large number of all cancer-related deaths were caused by bladder cancer [3]. Its success is expected to radically change the current practice of chemotherapy, as well as greatly improve the quality of life of patients, sustained drug exposure and possible reduction in health care cost [4]. One of the main reasons for the failure of current chemotherapies was linked to the lack of adequate accessing of drugs to all cancer cells, leaving some of these cancer cells alive. Unfortunately, the majority of cytotoxic drug, especially those with superior and broad spectrum of anticancer activity such as paclitaxel, have poor bioavailability.

Intravesical administration of drug provides the advantage of local exposure to the drug. However, variable results are obtained among patients leading to think of new applications to enhance the outcomes. Studies have shown that the incomplete responses of intravesical treatment with mitomycin c (MMC) and doxorubicin (the most commonly used drugs for intravesical treatment) are in part due to their limited penetration through bladder tissue [5, 6]. For instance, Au et al [7] have shown in randomized phase III clinical trial that manipulated the pharmacokinetics (PK) of MMC by increasing the dose and stability, resulted in improved efficacy (increased tumor recurrent-free frequency and time).

Nanoparticles are able to preferentially accumulate at tumor sites by extravasation through the leaky vasculature of tumor sites via the well-known enhanced permeability and retention (EPR) effect. SNEFs possess considerable potential for use in drug delivery as the systems are highly biocompatible have long life-span, protect the loaded drugs from inactivation and high amount of drugs can be loaded into a small volume of SNEFs. These properties, in turn, assure the delivery of sufficient dose using a limited volume of SNEFs as demonstrated in clinical and animal studies.

Nanodroplets within SNEFs are defined as particulate dispersions or droplets in aqueous media with a size in the range of 10-1000nm. Drugs can be dissolved, entrapped, encapsulated or attached to a nanoparticle matrix. Depending on the method of preparation, SNEFs can produce nanodroplets, nanospheres and nanocapsules [8]. The major goals in designing SNEFs as a delivery system are to control particle size, surface properties and to achieve site-specific action of the drug. As a drug delivery system SNEFs have several advantages including: passive and active drug targeting, increased therapeutic efficacy, lesser side effects and preservation of drug activity, enhanced cellular uptake and increased biological activity. SNEFs can be used for various routes of administration including oral, nasal, parenteral and intra-ocular. The bladder intravesical route for nanoemulsion chemotherapy preparations is promising.

SNEFs have limitations as the solubilisation depends on the solvent capacity of the excipients used in the formulation design. The solubilisation can be enhanced in self-nanoemulsifying formulations (SNEFs) by addition of hydrophobic material (Polar oils) that provides more space for solubilisation of poorly soluble drugs [9]. SNEFs of some drugs such as paclitaxel [10], doxorubicin [11], and curcumin [12] have been explored with enhanced therapeutic potential.

SNEFs vary from a simple oil solution to complex mixtures of oils and surfactants. It covers a wide range of formulation types, such as solutions, emulsion, self-emulsifying formulations and micellar systems and suitable for poorly soluble drugs. Self-nanoemulsifying formulations have many advantages over other conventional drug delivery systems. Mainly, fine oil droplets of SNEFs increases stability of the drug and provide a large interfacial area for partitioning of the drug between oil and water. There are mainly two types of SNEFs reported, which include Self-microemulsifying systems and Self-nanoemulsifying systems and their differences in the characteristic features [13], [14], [15].

Paclitaxel is diterpenoid, poly-oxygenated pseudo alkaloid anticancer drug isolated from the bark of the Pacific yew tree (*Taxus brevifolia*) [16, 17]. It is an extremely hydrophobic drug and practically considered as water-insoluble [18]. [17] [19]. Paclitaxel acts by promoting and stabilizing the polymerization of microtubules thereby, inhibiting cell replication and blocking cells in the late G2-mitotic phase of the cell cycle and its mechanism of action is unique and characteristic to it and its congener toxoids [19]. Moreover, paclitaxel may exert its anticancer effect through other different mechanisms such as inhibition of tumor angiogenesis, stimulation of tumor necrosis factors, and induction of cytokines and tumor-suppressor genes [20]. Paclitaxel is considered the backbone of female cancer chemotherapy such as breast cancer and ovarian cancer [18]. It is also approved for refractory AIDS-related Kaposi's sarcoma, non-small-cell lung carcinomas, and advanced esophageal cancer [18].

The main problem associated with Taxol® chemotherapy is the severe hypersensitivity reactions which necessitate the administration of high-dose glucocorticoids, H1 antagonist antihistamines and H2 antagonist as a premedication protocol for all patients receiving Taxol® [21].

Here, we proposed to develop and evaluate self-nanoemulsifying formulations (SNEFs) as a carrier for paclitaxel used for intravesical chemotherapy of the bladder cancer. One important property of SNEF is the formation of colloidal-sized clusters in solutions, known as micelles, which express significance in pharmaceutical formulations because of their ability to enhance the solubility of poorly soluble drugs in water. Additionally, numerous drug delivery and drug targeting systems have been developed in an attempt to prevent harmful side effects, to decrease drug degradation and loss, and to enhance drug bioavailability.

Several studies have attempted to improve the penetration of paclitaxel injected into the bladder by using solvents, surfactants and solid nanoparticles. The current study aimed to develop non-toxic nanoemulsion carriers of paclitaxel and to evaluate their ability to serve as a tool increasing the stability and solubility of paclitaxel utilizing the drug intravesical administration into the bladder. As far as we know, there is no previous study evaluating SNEFs as a tool for intravesically administered paclitaxel into the bladder.

2. Materials and Experimental methods

Materials

All chemicals/materials used in this study were obtained from well-known commercial suppliers and used without further purification. Paclitaxel was obtained from BioTrend chemicals AG, Zurich, Switzerland. Kollisolv 70 (K70) (medium chain triglyceride, C_8-C_{10}), Imwitor 988 (I988) (medium chain mono- and di-glycerides) were obtained from Sasol, GmbH, Werk Witten, Witten-Germany. Cremercoor® MCT 70/30 (M70) (caprylic/capric acid triglycerides (70% caprylic C8, 30% capric C10) was purchased from Cremor Oleo, GmbH, Germany. Capmul MCM (CM) (Mix of Mono-

80% & diglycerides C_{8-10}) was obtained from Abitec Corporation, Germany. The non-ionic surfactants HCO-30 (Polyoxyethylene hydrogenated castor oil, HLB - 11) was a gift from Nikko Chemicals Co. (Tokyo, Japan). All other chemicals used in the preparation of SNEFs were obtained from Sigma-Aldrich, UK unless specified otherwise. Glass culture tubes 13×100 mm (Pyrex®, screw cap) (Corning, USA) were used for fraction collecting and mixed using a Vortex mixer. A thermostated water bath (Ratek Instruments Pty. Ltd., Boronia, Victoria) was used to incubate all the sample containing tubes at two different temperatures (22ºC and 37ºC). Phase transitions and liquid crystalline phase were identified using the Allen viewer, which was fitted with cross-polarizing filters. High purity Milli-Q water was obtained through a Milli-Q pure lab flex (ELGA by Veolia water, UK).

Experimental methods

SNEFs formulation design

A number of primary and secondary lipid oils from various manufacturers (Table 1) with known surfactants were mixed to develop SNEFs within lipid-based formulations. The formulations were prepared by a simple two steps preparation method using varying concentrations of single or blend of two oils, and surfactant (% weight). In the primary step, oil mixture was prepared with oils of different kinds from various manufacturers. Details of the chemical compositions of the materials are listed in Table 1. Then a surfactant was added to the primary mixture of oils at various ratios. The homogeneity of final mixture was achieved by vortexing at room temperature. The anhydrous formulation mix was kept in an airtight 3 mL glass vial prior to use.

184 Afterwards, the most suitable SNEFs formulations were investigated carefully using
185 paclitaxel.

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187 **Table 1**: Description of the lipid excipients and their chemical compositions based on
188 their suppliers, functional group, or their role in the formulation

Abbreviation	Name	Function and Chemical composition
K70	Kollisolv MCT 70	Primary Oil, Medium chain triglyceride [C_8-C_{10}]
M70	Cremercoor MCT70/30	Primary Oil, Medium chain triglyceride [C_8-C_{10}]
I988	Imwitor 988	Secondary Oil, Medium chain mono and diglycerides [higher C_8]
I742	Imwitor 742	Secondary Oil, Medium chain mono-, di- and triglycerides, [C_6-C_{10}]
M812	Miglyol 812	Primary Oil, Medium chain triglycerides, [higher C_8]
M810	Miglyol 810	Primary Oil, Medium chain triglycerides, [higher C_{10}]
CM	Capmul MCM	Secondary Oil, Medium chain mono & diglycerides, [higher C_{10}]
TO	TO106V	Surfactant, PEG 6 sorbitan monooleate, HLB-10
HCO30	Hydrogenated castor oil	Surfactant, POE-30-hydrogenated castor oils, HLB-11
T85	Tween 85	Surfactant, Polyoxyethylene sorbitan trioleate, HLB-11.5
KrEL	Kolliphor EL	Surfactant, Polyoxyl 35-hydrogenated castor oil, HLB-14.5
CrRH40	Cremophor RH40	Surfactant, Polyoxyl 40-hydrogenated castor oil, HLB- 16

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Studies of equilibrium phase behavior

Phase equilibria within ternary systems are most conveniently represented by a triangular phase diagram which delineates the phase regions enabling to select suitable combinations of the three components to produce stable emulsions or clear isotropic solubilized systems as required. In this case phase diagrams were used to represent all the possible mixtures of oil, surfactant and water systems. When oils were blended to modify the polarity of the oil phase, the oil mixture ratio was constant allowing similar phase diagrams to be constructed.

The phase diagrams were investigated at two different temperatures (22 and 37°C) using the following methods [22]. Briefly, the samples were prepared at different weights and one-phase compositions were identified by visual observation. In cases where needed, samples were left at particular temperature and observed for several weeks in order to estimate the long term stability of phases and, also, to obtain an improved separation. Different liquid crystalline phases were identified using optical viewer (Allen viewer) with the sample viewed between crossed polarizers. The following systems were studied for the triangular phase diagrams using Oil-Surfactant-Water mixture.

System 1: K70: I988 (7:3)/KrEL/Water

System 2: M70: CM (7:3)/HCO30/Water

The preparation method of SNEFs and characterizations

a) Preparation of primary mixtures of the formulation

In the experiments the primary sample was prepared for those systems, where the blending of two oils has been used with the surfactant. For instance, the mixture of

K70 and Imwitor 988 (7:3) was prepared for K70: I988 (7:3)/KrEL/Water system as a primary mixture by weighing on a model Bp 210S Sartorius balance. The mixture was mixed carefully with a vortex mixture to ensure the uniformity. These mixture ratios of two oils would remain constant for the secondary mixture.

b) Preparation of secondary mixtures of the formulation

Once the primary mixture was complete, the secondary mixture was prepared by adding various weight percentage of non-ionic surfactants. According to different oil/surfactant ratios 8 to 11 sample mixtures prepared in the secondary system were accurately weighed into a 20 ml glass beaker followed by vortex mixing. The mixture of oil and surfactants were used to prepare emulsions under conditions of gentle agitation at a controlled temperature.

c) Preparation of ternary mixtures of the formulation

The ternary mixture was prepared by adding accurately water (%, w/w) into the secondary mixture (oil/surfactant), which were weighted into screw-capped glass tubes with water-tight closures. For 8-11 sample mixtures in the secondary formulation, 80-100 mixtures were prepared at different weights in a number of glass tubes during the ternary formulation. The data points were determined in thermostated samples with the aid of a water bath at 22°C, and 37°C temperatures. All Phase transition was determined by placing the samples between cross polarizers. The number and types of phases were recorded.

d) The Visual assessment of SNEFs

A visual test to assess the self-emulsification properties of the current SNEF formulations was initially adapted with a minor change from our previous studies [23].

Briefly, within the scope of the present investigation, visual test was important to avoid trial and errors. To screen each formulation, spontaneity, dispersibility and appearance were taken into considerations after aqueous dilution of the anhydrous formulations. Formulation (100mg) was dropped into 10 mL (w/v) (1 in 100 dilution factor) of distilled water and agitated gently at room temperature. The emulsion time (referred as spontaneity) and also the progress of emulsion droplets were observed against time. Spontaneity was considered as "efficient" when the droplets of formulation spread in distilled water effortlessly to form emulsion in $\leq$ 1 minute. On the other hand, dispersibility was defined as 'good' when the formulation droplets were completely dispersed in distilled water without floating particles. To confirm the efficiency of the formulations further characterization was also performed in considering their appearance. The appearance of the formulations was taken as milky, hazy or transparent according to the degree of clarity after aqueous dilution.

e) The Particle size distribution of the SNEFs formulation

The Zetasizer nano-25 (Malvern Instruments Limited, United Kingdom) was used in this study in standard autosizer mode to assess the size distribution of particles at the SNEF after aqueous dispersion. The representative self-emulsifying formulations were diluted in distilled water (1:1000 v/v) and mixed for 1 minute to ensure its disparity before testing. The particle size analysis was performed on 1 mL sample volumes using glass cuvettes. Measurements were performed at room temperature, with 10-min runs, using a 400 mm aperture. Values were calculated by finding the mean of three separate measurements for each formulation. Results were expressed as the Z-average mean (the harmonic intensity averaged particle diameter. The cuvette was used to fill diluted

samples in the sample compartment and the data were collected for 10 times. All experiments were carried out in triplicates.

f) Influence of dilution with water for Injection (WFI) or saline (NaCl 0.9%) on SNEFs stability

Particle size distribution and visual assessment were performed as described above of the emulsions after dispersion in serial dilution in WFI or saline to ensure their stability at various dilution rates. The representative self-emulsifying formulations were diluted in a ratio of 1:100 to 1:10000 v/v (SNEF:WFI/0.9% NaCl) and mixed for 1 min before testing.

g) Influence of pH on SNEFs stability

Particle size distribution and visual assessment were performed as described above of the emulsions after dispersion in aqueous dilution (1:1000 v/v, SNEF:0.9% NaCl). Salt solutions were buffered at various pH range (4 to 8.8) in order to ensure their stability at conditions similar to that in the urinary bladder. The representative self-emulsifying formulations were diluted in a ratio of 1:10 to 1:10000 v/v (SNEF:WFI) and mixed for 1 min before testing.

h) SNEFs loading with paclitaxel

The paclitaxel-loaded SNEFs at the concentration of 50 µg/ml were prepared by adding 2 µl of paclitaxel (250 mg/ml) into 98 µl SNEF and mixed well before the resultant solution was added into cell culture media at ratio 1:100 v/v. The paclitaxel-loaded SNEFs at the concentration of 250 µg/ml were prepared by adding 2 µl of paclitaxel (250 mg/ml) into 98 µl SNEF and mixed well before the resultant solution was added into cell culture media at ratio 1:20 v/v. The representative self-emulsifying

formulations were diluted in a ratio of 1:10 to 1:10000 v/v (SNEF:WFI) and mixed for 1 min before testing. Particle size distribution and visual assessment were performed as described above.

Chemosensitivity studies

a) Materials and chemicals

MCF7 Human Breast cancer cells were obtained from American Type Culture Collection (ATCC), USA. DLD1 colorectal cancer cells were a kind gift from Dr. Ahmad Al-Jada'a's lab (King Saud bin Abdulaziz University for Health Sciences, Riyadh, KSA). Hanks Balanced Salt Solution (HBSS), Trypsin/EDTA solution, RPMI 1640 medium and its' components were obtained from Invitrogen, USA

b) Cell culture condition and maintenance

Cancer cell lines were kept frozen in liquid nitrogen for long storage. When required cells were briefly warmed to 37°C before transfer to pre-warmed 10 ml complete RPMI 1640 medium consisted of 1 mM Sodium pyruvate, 2 mM L-glutamine and 10% Fetal Bovine Serum and centrifuged for 5 min at 1700 rpm (250 *g*). Cells were re-suspended in complete medium. Cells were then placed into 75ml flask left to adhere overnight in a 5% CO_2 enriched humidified incubator at 37°C. During the project, cells were maintained routinely as a monolayer in complete RPMI 1640 medium in humidified incubator at 37 °C in a CO_2 enriched atmosphere (5%). Cells were left to grow in sterile T75 cell culture flasks until reached approximately 80% confluence. When required, supernatant was removed and monolayer cells were washed twice in Hanks' Balanced Salt Solution (HBSS) before adding 5 ml of trypsin/EDTA solution for 10 minutes. After

obtaining a single-cell suspension, 10 ml of medium was added to neutralize the trypsin. Cell-suspension was then centrifuged at 250 *g* for 5 minutes. The resultant cell pellet was re-suspended in 10 ml complete RPMI medium and counted under inverted microscope using heamocytometer.

c) Measurement of SNEFs toxicity on cultivated monolayer cancer cells using Trypan Blue

Trypan Blue is a stain used in dye exclusion procedures for viable cell counting. This method is based on the principle that Trypan Blue does not enter viable cells (remain clear), whereas it enters and stains the dead cells (stained blue). Briefly, cancer cells (2 x 10^5 cells) were exposed to different SNEFs and incubated for one hour in a 5% CO_2 enriched humidified incubator at 37°C. After that, cells were centrifuged and re-suspended in 200 µl complete medium followed by 200 µl of 0.4% Trypan Blue solution (w/v) and mixed well. Immediately after that, 10 µl of the suspension mixture was transferred into each chamber of a haemocytometer. Viable (non-stained) and non-viable cells (stained blue) in each 1 mm square were counted and at least 10 counts were performed. The percentage cell viability of cells was then determined using the following calculations:

% cell viability = (number viable cells / number of total cells) x 100%

d) MTT assay

MTT assay to be performed as described in the literature [24] 20 µl of MTT solution (5 mg/ml) to be added into each well and incubated in the dark at 37^{o}C for 4 hours. The supernatant was removed and formazan crystals were dissolved in DMSO (150 µl). The absorbance was read immediately at 540 nm in multi-well plate

spectrophotometer (BioTek, USA). The average absorbance in the control wells was taken as 100% survival, and the IC_{50} values were defined as the drug concentrations that inhibited the cell growth by 50%.

e) Measurement of drugs toxicity on cultivated monolayer cancer cells following 96-hour exposure

Cancer cell lines were seeded into 96 well plates ($3x10^3$ cell/well) and left to adhere overnight at 37 °C in a 5% CO_2 enriched humidified incubator. The medium was then replaced with fresh one containing a suitable concentration range of drug (paclitaxel) Cells were then incubated in humidified incubator at 37°C for 96 hours before the MTT assay was performed as described above.

f) Measurement of drug toxicity on cultivated monolayer cancer cells following one-hour exposure

Cancer cell lines were seeded into 96 well plates (3×10^3 cell/well) and left to adhere overnight at 37 °C in a 5% CO_2 enriched humidified incubator. The medium was then replaced with fresh one containing a suitable concentration range of drug (paclitaxel). Cells were then incubated in humidified incubator at 37°C for one hour. After the completion of the time course, cells were washed twice using HBSS and substituted by fresh medium containing no drug. The plates then were incubated for 95 hours before the MTT assay was performed as described above.

g) Measurement of paclitaxel-loaded SNEF toxicity on cultivated monolayer cancer cells following one-hour exposure

Cancer cell lines were seeded into 96 well plates (3×10^3 cell/well) and left to adhere overnight at 37 °C in a 5% CO_2 enriched humidified incubator. Cells were then exposed to medium containing a suitable range of paclitaxel-loaded SNEFs (diluted by RPMI medium). Cells were then incubated in a humidified incubator at 37°C for one hour. After the completion of the time course, cells were washed twice using HBSS and substituted by fresh medium containing no drug. The plates then were incubated for 95 hours before the MTT assay was performed as described above.

Statistical Analysis

Data were analyzed using SPSS 22.0 (IBM Corp., Armonk, NY, USA). Impact on inhibition was compared using One Way ANOVA, and the significant differences were decided at a confidence interval of 95% and $P < 0.05$. In addition, Tukey Post Hoc statistical test was employed to produce pairwise comparisons among treatments.

3. Results and discussion

SNEFs Preparation and Physical Evaluation

A) Construction of Ternary Phase Diagram

The ternary phase diagrams were mapped at different temperatures (22°C and 37°C) to select efficient self-nanoemulsifying regions. In addition, the concentration of oil and surfactant was determined to develop stable model drug-loaded SNEFs. Figure 1a and 1b show representative constructed phase diagrams of oil-mix (K70:I988), surfactant (KrEL) and water at 22°C and 37°C.

B) Selection of self-nanoemulsifying formulations from phase diagrams and their assessment

The phase diagram constructed at 22°C illustrates that 50 % oil onward with surfactants are able to solubilize significantly large amount of water (Figure 1a & 2a). These anhydrous mixtures mostly produce the nanoemulsifying formulations. Low oil quantity with surfactant produces crude emulsion which will not be targeted for scope of the current work. Hence, the L2 region (oil continuous) L1 (water continuous) regions are considered to be the nanoemulsifying regions. Different concentrations of oil such as 10%, 20%, 30%, 35%, 40% w/w), was utilized for preparation of effective nanoemulsion. This helped in selecting of various formulations covering the entire nanoemulsion area of phase diagram.

The phase diagram of the same components at 37°C did not show very different phase regions (Figure 1b & 2b). Only the liquid crystalline phase (LC) was narrowed down to L2 phase. The selected SNEFs from the phase diagrams emulsifies less than a minute, giving transparent and or bluish appearance nanoemulsion. The prepared SNEFs are proved to show the thermodynamic stability due to the adsorption of surfactant at the interface that decreases the interfacial energy.

(C) Visual assessment of the SNEFs

Visual observation is a plausible mean of assessment to the experienced eye in order to differentiate efficient and poor formulations since most of these formulations are expected to produce nano-sized droplets upon dispersion. The following factors were considered for visual assessments to optimise SNEFs formulation: homogeneity, time for dispersion and appearance upon dilution with water. The dilution ratio of 1:500 (formulation: water) was maintained. In the current study, efficient formulations are defined when homogeneous dispersion (transparent/bluish appearance in Table 2) is achieved in less than 1 min. As shown in Table 2, all representative formulations except

SNEF 6, 15 & 18 (non-dispersible in aqueous media) were found to be efficient and were used for further optimization.

Table 2: Visual assessment of dispersions formed by different formulation systems under self-emulsification conditions (NA denotes "not available")

No	Formulation Name (%W/W)	Dispersibility	Appearance after dispersion	Efficient SNEF
SNEF 1	K70:I988(7:3)/KrEL[70/30]	~10 Sec	Turbid	√
SNEF 2	K70:I988(7:3)/KrEL[50/50]	~10 Sec	Transparent	√
SNEF 3	K70:I988(7:3)/KrEL[30/70]	~1min	Transparent	√
SNEF 4	K70:I988(7:3)/KrEL[20/80]	<2min	Transparent	√
SNEF 5	M70:CM(7:3)/HCO-30[50/50]	~10 Sec	Bluish	√
SNEF 6	M70:CM (7:3)/TO [70/30]	NA	2 phase solution	X
SNEF 7	M70:CM (7:3)/TO [50/50]	<2min	Turbid	√
SNEF 8	M70:CM (7:3)/TO [20/80]	~10 Sec	Turbid	√
SNEF 9	M 812: I 988 (7:3)/T85[40/60]	~10 Sec	Turbid	√
SNEF 10	M812 : I988 (7:3)/T85[60/40]	~10 Sec	Hazy	√
SNEF 11	M812 : I988 (7:3)/T85[90/10]	~10 Sec	Turbid	√
SNEF 12	M812 : I988 (7:3)/KrEL[50/50]	<2min	Transparent	√
SNEF 13	M812 : I988 (7:3)/KrEL[70/30]	~10 Sec	Transparent	√
SNEF 14	M812 : I988 (8:2)/KrEL[90/10]	~10 Sec	Turbid	√
SNEF 15	M812 : I988 (9:1)/KrEL[70/30]	NA	2 phase solution	X
SNEF 16	M810:I742(7:3)/CrRH40[50/50]	<2min	Transparent	√
SNEF 17	M810:I742(7:3)/CrRH40[70/30]	~10 Sec	Transparent	√
SNEF 18	M810:I742(9:1)/CrRH40[90/10]	NA	2 phase solution	X
SNEF 19	M810:I742(8:2)/CrRH40[90/10]	~10 Sec	Turbid	√

(D) the particle size distribution of the SNEFs formulation

The selection of the optimized SNEFs was based on the stability, reproducibility of SNEFs size after dispersion, appearance and also single sized population. The SNEF was considered stable if retained its characteristics after dispersion for at least 24 h at room temperature. Reproducible SNEFs are those generate the same droplet size ± 20% in each time after dispersion in different days over a period of one week. Appearance is accepted regardless if it is transparent, hazy or even turbid with the condition that the SNEF retains its stability and gives a single sized population peak in the Zetasizer analysis. SNEFs with 2 phases appearance are considered unacceptable (SNEF 6, 15 & 18). Interestingly, none of the SNEFs sized ≥100 nm did have these conditions. It may be worth mentioning that formulations with turbid dispersion appearance were heterogonous with the droplets sizes ranging between 163.6 and 551.6 nm.

Table 3: Droplet size distribution of different SNEFs within lipid-based formulations. Data are presented as mean only (n=3, SD < 2%)

No	Formulation Name (%W/W)	Average particle size (nm) 1/100 dilution in WFI	PDI
SNEF 1	K70:I988(7:3)/KrEL[70/30]	95.64	0.247
SNEF 2	K70:I988(7:3)/KrEL[50/50]	30.1	0.236
SNEF 3	K70:I988(7:3)/KrEL[30/70]	22.2	0.161
SNEF 4	K70:I988(7:3)/KrEL[20/80]	18.9	0.200
SNEF 5	M70:CM(7:3)/HCO-30[50/50]	44.3	0.176
SNEF 6	M70:CM (7:3)/TO [70/30]	365.0	0.241
SNEF 7	M70:CM (7:3)/TO [50/50]	403.0	0.257
SNEF 8	M70:CM (7:3)/TO [20/80]	394.8	0.352
SNEF 9	M 812: I 988 (7:3)/T85[40/60]	163.6	0.171
SNEF 10	M812 : I988 (7:3)/T85[60/40]	95.1	0.275

SNEF 11	M812 : I988 (7:3)/T85[90/10]	551.6	0.042
SNEF 12	M812 : I988 (7:3)/KrEL[50/50]	32.4	0.012
SNEF 13	M812 : I988 (7:3)/KrEL[70/30]	62.4	0.194
SNEF 14	M812 : I988 (8:2)/KrEL[90/10]	281.2	0.484
SNEF 15	M812 : I988 (9:1)/KrEL[70/30]	234.3	0.244
SNEF 16	M810:I742(7:3)/CrRH40[50/50]	37.3	0.064
SNEF 17	M810:I742(7:3)/CrRH40[70/30]	59.5	0.047
SNEF 18	M810:I742(9:1)/CrRH40[90/10]	295.7	0.538
SNEF 19	M810:I742(8:2)/CrRH40[90/10]	200.7	0.026

Their sizes showed in Table 3 represent the average particle size. For instance, F15 was dispersed into more than one size group ranged between 50 nm and 6000 nm. Another observation is that a majority SNEFs are forming a viscous jelly when diluted at low water ratio (data not shown). Based on this observation, we evaluated the SNEFs dispersion at a dilution ratio 1:100. SNEFs at this ratio as would have good dispersion stability as well as relatively high drug capacity. Representative intensity graphs are presented in Figure 3.

Intravesically administered SNEFs will also be exposed to urine in which the salt levels (especially sodium) and pH can vary greatly. In order to evaluate the stability of dispersed SNEFs in such conditions we used normal saline to mimic the maximum sodium levels in the urine. We also dispersed the selected SNEFs in normal saline buffered at different pH similar to possible urine pH levels. Interestingly, only a negligible change was observed in the size of particles after dispersion in normal saline,

regardless the pH level (see Table 4 and 5). Whereas all selected SNEFs were appeared transparent after dispersion.

Table 4: Visual assessment of dispersions formed by selected formulation systems under self-emulsification conditions in cell culture medium or normal saline.

No	Formulation Name (%W/W)	Particle size (nm) 1/100 dilution (PDI)	
		Medium	0.9% NaCl
SNEF 2	K70:I988(7:3)/KrEL[50/50]	32.39 (0.142)	31.09 (0.210)
SNEF 3	K70:I988(7:3)/KrEL[30/70]	21.74 (0.108)	24.8 (0.228)
SNEF 4	K70:I988(7:3)/KrEL[20/80]	20.49 (0.217)	18.22 (0.112)
SNEF 5	M70:CM(7:3)/HCO-30[50/50]	42.52 (0.145)	43.68 (0.181)
SNEF12	M812 : I988 (7:3)/KrEL[50/50]	32.22 (0.148)	31.46 (0.076)
SNEF13	M812 : I988 (7:3)/KrEL[70/30]	40.69 (0.111)	56.86 (0.107)
SNEF16	M810:I742(7:3)/CrRH40[50/50]	40.08 (0.135)	38.88 (0.208)
SNEF17	M810:I742(7:3)/CrRH40[70/30]	62.7 (0.104)	61.03 (0.021)

Table 5 Visual assessment of dispersions formed by selected formulation systems under self-emulsification conditions in 0.9% NaCl at different pH.

No	Formulation Name (%W/W)	Particle size (nm) after 1/100 dilution with 0.9% NaCl at different pH (PDI)		
		pH 4	pH 7	pH 8.8
SNEF3	K70:I988(7:3)/KrEL [30/70]	22.91 (0.117)	23.81 (0.216)	18.1 (0.051)
SNEF5	M70:CM(7:3)/HCO-30 [50/50]	46.96 (0.187)	45.22 (0.205)	41.28 (0.218)
SNEF12	M812 : I988 (7:3)/KrEL [50/50]	31.23 (0.081)	31.27 (0.086)	29.29 (0.067)
SNEF13	M812 : I988 (7:3)/KrEL [70/30]	55.14 (0.132)	49.84 (0.08)	50.87 (0.154)
SNEF16	M810:I742(7:3)/CrRH40[50/50]	40.08 (0.135)	38.88 (0.208)	38.88 (0.208)
SNEF17	M810:I742(7:3)/CrRH40[70/30]	61.61 (0.007)	61.01 (0.065)	57.69 (0.01)

Changing the dilution factor from 1:20 to 1:10000 also did not have a major impact on the size of the dispersed SNEFs regardless if the dilution was made gradually by serial dilution or instantly from SNEFs into desired solution volume (Data not shown). The results presented in Table 5 show good stability of some developed SNEFs after dilution with WFI, media or normal saline. Different pH conditions mimicking the pH inside the bladder also had a negligible effect (Table 5). These results adhere well with that reviewed by Anton and Vandamme [25]. This is important as the urine collection in humans is assumed to be 1 ml per minute, which gives a huge dilution of the preparation after intravesical administration of 40 ml of the drug for 60 minutes.

2) SNEF effects on cancer cells monolayers

A) Evaluation of Toxic effects of unloaded-SNEFs on cancer cells

Trypan blue is known to stain dead (necrotic) cells as it cannot pass through the intact cellular membrane. Many chemicals may cause cell necrosis leading to cell membrane rupture. We here investigated whether or not our developed SNEFs may cause cell necrosis within 60 minutes of exposure. The result in Table 6 shows that SNEFs were not toxic to DLD1 cells after one-hour exposure to selected SNEFs. In addition, SNEF 5, and SNEF 12, were also not toxic on CRL1772, T24, HT1197 and RT4 bladder cancer cells (data not shown). This indicates that SNEFs are relatively safe to be used in intravesical installation settings.

Table 6 The toxicity of non-loaded SNEFs 1/100 dilution on DLD1 cells using Trypan Blue. The results represent the mean survival percentages of SNEFs-treated cells compared to the mean percentage of non-treated control.

No	Formulation Name (%W/W)	% survival
SNEF2	K70:I988(7:3)/KrEL[50/50]	96.85
SNEF3	K70:I988(7:3)/KrEL[30/70]	104.66
SNEF4	K70:I988(7:3)/KrEL[20/80]	103.67
SNEF5	M70:CM(7:3)/HCO-30[50/50]	98.77
SNEF12	M812 : I988 (7:3)/KrEL [50/50]	93.51
SNEF13	M812 : I988 (7:3)/KrEL [70/30]	94.91
SNEF16	M810:I742(7:3)/CrRH40[50/50]	96.07

476

477

478 ***B) Chemosensitivity of DLD1 monolayer to paclitaxel following 1 and 96-hour drug***
479 ***exposure***

480 Drugs administered into the bladder by intravesical installation are kept inside
481 the bladder usually for 1 hour. This short time course requires that drugs to be
482 intravesically administered into the bladder should exhibit high anticancer activity at
483 relatively low concentrations. Otherwise, these drugs may not be able to reach the
484 desired pharmacologically active concentrations within the bladder cancer tissue. Figure
485 4 showed the chemosensitivity of DLD1 cells exposed to paclitaxel. As expected, the
486 dose-response curves were shifted to the right when the exposure time was reduced to 1
487 hour. The IC_{50} values for paclitaxel after 96-hour exposure was 43.9± 14.2 nM. When
488 cells were exposed to paclitaxel for 1 hour the IC_{50} value was found to be 3.55± 2.75
489 µM. These results indicate that paclitaxel is a good candidate for intravesical treatment
490 of bladder cancer. The intravesically installed drug in the bladder is kept for a maximum
491 2 hours before the patient has to evacuate the bladder by urination. Therefore, great
492 concentrations of drug are required to be intravesically installed in the bladder in order
493 to reach sufficient therapeutic concentrations within the cancer tissue. However,

494 formulators should be aware of using high concentrations of drug as it may be
495 problematic due to the increased side effects and difficulties in preparing the dosage
496 formulations are expected.

497

498 ***C) Chemosensitivity of DLD1 cancer cells exposed paclitaxel-loaded SNEFs***

499 The results in Figure 5 also showed that DLD1 cells were less affected when
500 exposed to Paclitaxel-loaded SNEFs compared to paclitaxel alone. This may warrant
501 further investigation on the enzymatic and non-enzymatic degradation of SNEFs inside
502 the body. Rapid or slow degradation of SNEFs may influence the effectiveness of loaded
503 drugs. Based on these results, however, it could be speculated that SNEFs may improve
504 the penetration of drugs by reducing its metabolism which may give a greater chance of
505 treatments to reach deeper into the tissue [26]. Results also suggest that SNEFs may have
506 a beneficial role in reducing the site of administration toxicity seen by cytotoxic drugs
507 particularly such as paclitaxel [27].

508

509 ***D) Chemosensitivity of MCF7 cancer cells exposed to paclitaxel-loaded SNEFs***

510 In order to further investigate whether the chemosensitivity is modulated by the
511 cell type, SNEFs 5, 12 and 16 were selected for testing their toxicity on MCF7 breast
512 cancer cells. Interestingly, a different pattern was observed (Table 7). Although all
513 selected SNEFs were able to reduce the growth inhibition of paclitaxel after 1h or 96 h
514 exposure, SNEFs effects widely differed from each other. While SNEF 5 has increased
515 the IC_{50} only by approximately 4 folds, SNEF 16 loading has resulted in almost a loss

of activity. This clearly indicates that not only the cell type but also the formulation type itself has a critical role.

Table 7 The chemosensitivity of Paclitaxel-loaded SNEFs on MCF7 monolayer cells after exposure to 1 or 96 hours.

No	Formulation Name (%W/W)	MCF7 exposed to Paclitaxel Formulations	
		1 h exposure	96 h exposure
Unloaded	Un-loaded Paclitaxel	11.02 ng/ml	< 0.2 ng/ml
SNEF5	M70:CM(7:3)/HCO-30 [50/50]	40.91 ng/ml	0.75 ng/ml
SNEF12	M812:I988 (7:3)/KrEL [50/50]	281.31 ng/ml	19.16 ng/ml
SNEF16	M810:I742(7:3)/CrRH40[50/50]	34.72 µg/ml	30.38 µg/ml

The results from the chemosensitivity studies indicated that the increase of toxicity after the time of exposure with paclitaxel is somehow freed out of the nanodroplets. This freeing process may be due to SNEFs metabolism or simply by passive diffusion.

The observed reduction of paclitaxel toxicity when loaded into SNEFs indicates an efficient encapsulation of the drug. SNEFs may provide a physical barrier between the drug and the cells inhibiting the cellular uptake of the drug. This barrier, however, is expected to be temporary as the drug will diffuse over the time and the SNEFs will degrade during or after the penetration process.

All prepared SNEFs were able to enhance the solubility of paclitaxel as no precipitations were observed up to 24 hours after loading. Regardless of the type of ingredients, SNEFs with more oil percentage were able to form larger but less stable

nano-emulsions. SNEFs smaller than 100 nm are stable up to 48 hours at room temperature. Better stability of paclitaxel was achieved in paclitaxel-loaded SNEFs for 24 hours. The stability of paclitaxel after more than 24 hours of loading is however not investigated. The ability of SNEFs to retain their characteristics (droplet size and homogeneity) when exposed to variable salt and pH conditions similar to that in human bladder indicates that they may be used to utilize the delivery of drugs with poor water solubility such as paclitaxel.

4. Conclusions

All optimized SNEFs are demonstrated to be nontoxic *in vitro*, which mean that they are probably safe to be administered to human. The activity of paclitaxel indicates that the representative SNEFs are a better vehicle for water-insoluble drugs. Therefore, it is logical to conclude that the SNEFs prepared in this study may serve as a delivery tool for drugs insoluble in water.

The size and ingredient proportion of SNEFs played a major role in influencing the activity of paclitaxel as the larger SNEFs with higher oil proportion have inhibited the paclitaxel activities more than smaller SNEFs. Variation in the activity patterns between paclitaxel-loaded SNEFs indicates the involvement of SNEFs metabolism as a critical factor influencing the SNEFs drug delivery system. On the other hand, paclitaxel encapsulation may facilitate the penetration due to reduced cellular metabolism the drug. Further investigations are recommended to explore the oils and enzymes that are involved in the metabolism of SNEFs.

Finally, SNEF formulations may serve as an easy and low-priced option to formulate poorly-water-soluble drugs. To our best of knowledge, the oil in water SNEFs use in bladder intravesical paclitaxel delivery is a unique idea and has not been evaluated before for intravesical installation in the bladder.

5. Author Contributions

Qasem Abdallah: Conceptualization, Methodology, Funding acquisition. **Mohsin Kazi**: Formal analysis, Writing- Original draft preparation, Resources. **Mohammad Khaleel**: Data curation, Validation, **Ibrahim Al-Deeb**: Visualization, Investigation. **Abdel-Rahman Nasr**: Writing- Reviewing and Editing. **Roger Phillips**: Supervision.

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Declaration of Interest

The author reports no declarations of interest.

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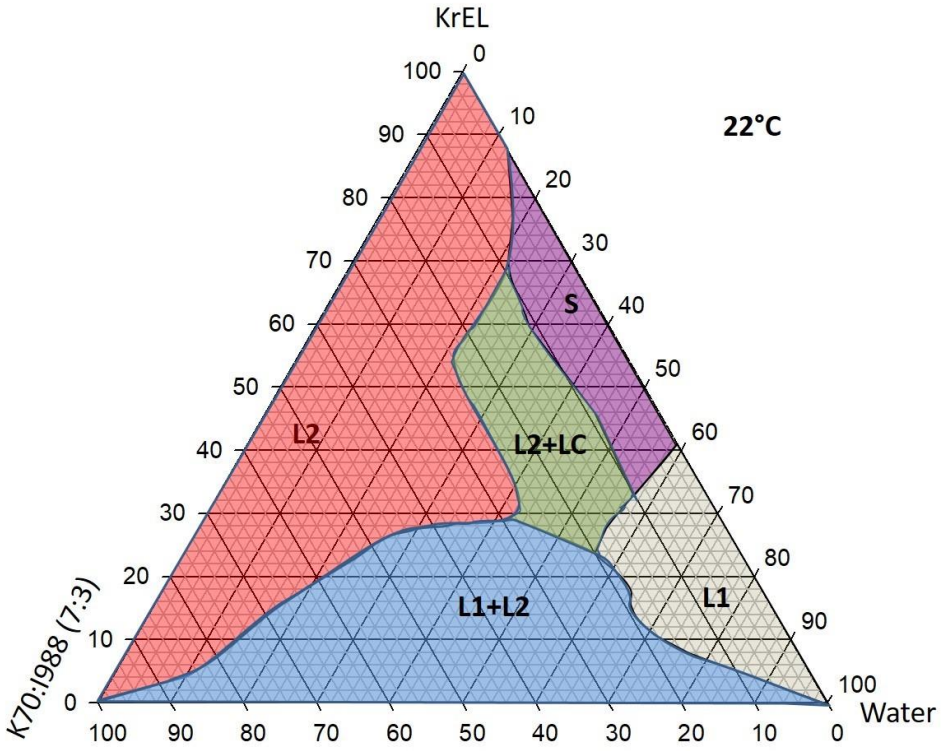
KrEL
22°C
S
L2
L2+LC
L1+L2
L1
K70:I988 (7:3)
Water
0
10
20
30
40
50
60
70
80
90
100

648