1 **Intracellular uptake of EGCG-loaded deformable controlled release liposomes for skin**
2 **cancer**

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23 **ABSTRACT**

24 Caucasian population groups have a higher propensity to develop skin cancer, and associated
25 clinical interventions often present substantial financial burden on healthcare services.
26 Conventional treatments are often not suitable for all patient groups as a result of poor
27 efficacy and toxicity profiles. The primary objective of this study was to develop a
28 deformable liposomal formulation, the properties of which being dictated by the surfactant
29 Tween 20, for the dermal cellular delivery of epigallocatechin gallatein (EGCG), a compound
30 possessing antineoplastic properties. The results demonstrated a significant ($p \leq 0.05$)
31 decrease in liposome deformability index (74 ± 8 to 37 ± 7) as Tween 20 loading increased
32 from 0 to 10 % w/w, indicating an increase in elasticity. EGCG release over 24-hours
33 demonstrated Tween 20 incorporation directly increased release from 13.7 % ± 1.1 % to 94.4
34 % ± 4.9 % (for 0 and 10 % w/w Tween 20 respectively). Finally, we demonstrated DilC-
35 loaded deformable liposomes were localised intracellularly within human dermal fibroblast
36 and keratinocyte cells within 2-hours. Thus it was evident deformable liposomes may aid
37 drug penetration into dermal cells and would be useful in developing a controlled-release
38 formulation.

39 **KEYWORDS:** Skin cancer; deformable liposomes; dermal release; controlled release;
40 elastic liposomes

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46 **1. Introduction**

47 Skin cancer is an increasing public health problem particularly in developed countries
48 [1]. Currently, 2-3 million non-melanoma skin cancers and 132,000 melanoma skin cancers
49 occur globally each year [2]. The large number of cases diagnosed present as a substantial
50 burden to healthcare services [3, 4, 2]. Despite the fact that the majority of skin cancers are
51 treatable, malignant forms of the cancer results in over 9,000 deaths annually worldwide [5].
52 Current treatment approaches are limited to local surgery to remove the tumour in addition to
53 topical treatments with cream formulations. However, surgical removal may not be suitable
54 for all patients whilst topical therapies are often linked with poor patient compliance
55 stemming from high dose frequency requirements and unpleasant side effects [6]. In addition,
56 topical treatments may cause skin irritation, weeping, cracking and blistering causing
57 discomfort and pain [7-9].

58 Strategies for cancer management are focused on chemoprevention and
59 chemoprotection. Existing anticancer agents often demonstrate poor safety profiles in
60 addition to unpleasant side effect profiles, and there is an urgent need for novel agents which
61 are both efficacious and possess a limited toxicity profile to non-malignant dermal tissue [10-
62 12]. One group of compounds that have gained interest recently as novel candidates for this
63 purpose are flavonoids, naturally occurring chemicals abundantly expressed in food and drink,
64 and in particular the green tea catechin, epigallocatechin gallatein (EGCG) which is
65 increasingly being exploited for its chemoprevention properties [13-15]. EGCG has been
66 found to affect specific biological processes that could be exploited as targets for the
67 prevention and treatment of cancer [16], and has been demonstrated to possess properties
68 associated with the induction of apoptosis [17], promotion of cell growth arrest by altering
69 the expression of cell cycle regulatory proteins [17], activation of killer caspases and the

70 suppression of oncogenic transcription factors [18, 19, 15] and pluripotency maintaining
71 factors [20]. However, the application of naturally occurring compounds as
72 chemopreventative and chemoprotective strategies for skin cancer management has so far
73 been received with limited success and this may be largely due to inefficient delivery systems
74 and limited oral bioavailability of promising agents [21-23]. Consequently, to achieve
75 maximum clinical efficacy, novel approaches are required to enhance compound
76 bioavailability, of which dermal delivery is particularly promising.

77 The principle function of mammalian skin is to offer protection from environmental
78 chemicals and xenobiotics [24]. The penetration of drugs across the skin is significantly
79 inhibited by the skin's inherent barrier properties [25] thus there is a need to develop carrier
80 systems to enhance penetrability. To fulfil this goal, when applied topically nanoparticle
81 mediated delivery systems (e.g. microemulsions, liposomes, ethosomes, deformable
82 liposomes and solid lipid nanoparticles), would benefit the direct dermal delivery of
83 compounds across the stratum corneum [26-28]. Additionally, such nano-scale structures are
84 capable of improving drug loading, enhancing systemic bioavailability, imparting a sustained
85 release profile and allowing targeted drug delivery [29, 30]. Furthermore, the topical
86 application of such carriers reduces the incidence of undesirable side effects arising from
87 systemic administration and enhances systemic absorption of drugs after topical application
88 with permeation enhancers which irreversibly disrupt the stratum corneum [29, 30].
89 Controversy however surrounds the use of conventional liposomes due to their large size
90 preventing skin penetration [31, 28, 32, 33], Cevc et al., [34] demonstrated that modification
91 of the chemical composition of the lipid bilayer so as to decease its Young's modulus
92 resulted in the formation of deformable liposomes. These are able to gain access to the viable
93 epidermis by overcoming the physical constraints imposed by the stratum corneum by
94 diminishing the membrane elastic energy required for the liposome to deform and fit through

an aperture size smaller than their original diameter following which reforming to their original shape [35, 36, 31]. By being able to change shape and volume at minimal energetic cost, these structures may penetrate across hydrophilic pathways of intact skin [37, 36]. Deformable liposomes often include additional components designed to make the membrane more liable to deformation and these are termed edge-activators, typically including surfactants such as Tweens, bile salts and Myrj [38]. The inclusion of this extra component destabilises the vesicle bilayers by reducing the amount of energy required to expand the interface allowing the liposome to become more elastic thus increasing the flux across the skin [38-40].

The primary aim of this study was to develop and characterise a deformable controlled release liposome formulation able to pass through the stratum corneum for targeting toward intracellular uptake into dermal cells. The objectives of the study were therefore to: (i) assess the impact of the edge-activator Tween 20 on liposomal formulation size; (ii) characterise resultant liposomes vesicle size, surface charge and encapsulation efficiency; (iii) quantify deformability of resultant liposomes using a deformability index (DI); (iv) assess release of EGCG; (v) assess the deformable liposome stability ; (vi) assess cellular toxicity of EGCG and (vii) assess intracellular uptake in human dermal fibroblast and keratinocyte cells.

2. Materials and methods

2.1 Materials

Soy Phosphatidylcholine (PC) was obtained from Avanti Polar Lipids. Cholesterol (grade, ≥99%), Tween 20 and EGCG (grade ≥95%, E4143) were obtained from Sigma-

118 Aldrich. All other reagents including methanol and chloroform were obtained from Fisher
119 Scientific. Ultrapure water was obtained from a Milli-Q purification system (Millipore,
120 Billerica, MA, US). Polycarbonate filter, pore size 50 nm, was obtained from Sigma-Aldrich
121 (WHA800309). Human dermal fibroblasts (HDFa) isolated from adult skin and all cell
122 culture reagents (Medium 106 and low serum growth supplement (LSGS) kit containing
123 supplemented medium containing foetal bovine serum, 2 % v/v, hydrocortisone 1 µg/mL,
124 human epidermal growth factor, 10 ng/mL, basic fibroblast growth factor, 3 ng/mL, heparin,
125 10 µg/mL; DMEM media supplemented with 1 % L-glutamine, 10 % FBS, 1 % Penicillin
126 Streptomycin and 0.25% amphotericin) were obtained from Life technologies (Carlsbad,
127 California, US). Immortalized human keratinocytes (HaCat) cells were a kind gift from Dr
128 Andrew Sanders (Cardiff China Medical Research Collaborative, Cardiff University, Henry
129 Wellcome Building, Heath Park, Cardiff, CF14 4XN). 4,6-diamidino-2-phenylindole (DAPI)
130 and 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DilC) was obtained
131 from ThermoFisher Scientific (D1306 and D282 respectively).

132 ***2.2 Methods***

133 *2.2.1 Preparation of deformable liposomes with or without an edge activator*

134 Liposomes were prepared by adapting the film hydration method established by
135 Bangham *et al*., (1965) [41]. Soy PC and cholesterol (16:8 µM) were dispersed in an organic
136 solvent mixture consisting of chloroform and methanol in a 9:1 (v/v) ratio in a round
137 bottomed flask [40, 38, 26, 42, 41]. Subsequently, the organic solvent was removed by rotary
138 evaporation for 5 minutes at 35 °C, followed by purging with nitrogen gas. The resultant dry
139 film residue was hydrated by the addition of 4 mL water containing edge activator (up to 10%
140 w/w of the formulation) and 1 mg of EGCG at a temperature above the transition temperature
141 of the phospholipid (between -7 to -15°C) [43] and vortexed for 5 minutes to form

142 multilamellar vesicles (MLV). The resulting particles were extruded 21 times through 100-
143 nm diameter polycarbonate membranes, using an Avanti Mini Extruder to produce
144 unilamellar vesicles. The formed liposomes were equilibrated for 30 min above their
145 transition temperatures (-15°C) before being subjected to further characterisation [44, 45, 43].

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147 *2.2.2 Deformable liposome characterisation*

148 The mean particle size and the polydispersity index (measurement of the level of
149 homogeneity of particle sizes) of liposomes were measured by dynamic light scattering
150 (DLS) using a Zetaplus (Brookhaven Instruments) following dilution with distilled water (1:4
151 ratio) to ensure intensity adjustment. A polydispersity value of < 0.2 indicates a homogenous
152 vesicle population, while polydispersity of > 0.3 indicates heterogeneity [46]. The particle
153 charge was quantified as zeta potential (ζ). Zeta potential was determined by photon
154 correlation spectroscopy using a Zetaplus (Brookhaven Instruments). The samples were
155 diluted three-fold and assessed in triplicate.

156 *2.2.3 HPLC-UV detection of EGCG*

157 Detection of EGCG was assessed using reverse phase HPLC methodology. A Waters
158 Alliance separation module HPLC with UV detection was utilised at an operating wavelength
159 of 275 nm [47] with a Waters X select column (5 µm C18 4.6 x 150 mm, 186005290), with a
160 10 µL injection volume. The mobile phase comprised of a 70:30 ratio of 0.1% TFA in water
161 to methanol at a flow rate of 1 mL/min. Stock solutions and standard solutions of EGCG
162 were prepared with both water and ethanol ranging from 0.5-500 µg/mL. A final calibration
163 curve with an R^2 of 0.997 and linear equation of $y = 1 \times 10^7 \cdot x$ was obtained.

164 *2.2.4 Entrapment efficiency of EGCG*

165 The entrapment efficiency of EGCG loaded deformable liposomes was determined by
166 centrifuging samples and quantifying the EGCG in the supernatant. Samples were centrifuged
167 at 18,000 rpm for 30 min at 4°C in an Optima^{tm} MAX-XP ultracentrifuge to separate the
168 incorporated drug from the free drug. The supernatant was then analysed using HPLC to
169 determine the encapsulation efficiency of EGCG in liposomal formulations (Equation 1):

170 $$E = \frac{D_t - D_S}{D_t} \times 100\% \quad (1)$$

171 where E is the encapsulation efficiency (%), D_t is the total drug content (mg) and D_s is drug
172 content in supernatant (mg).

173 *2.2.5 Assessment of liposomal deformability*

174 To assess the deformability of formulated liposomes, a liposome suspension (6 mL)
175 consisting of a 16:8 micromolar ratio of PC to cholesterol formulated with up to 10% w/w of
176 Tween 20 solution (diluted 3 fold), was passed through a polycarbonate filter of 50 nm pore
177 size using a syringe driver (Cole Parmer, UK) set at 0.6 mL/min for 10 min. The mean
178 particle size and the polydispersity index of liposomes were subsequently quantified by DLS,
179 before and after filtration, to assess the ability of formulated liposomes to regain their size
180 after having been forced through a pore size smaller than their original diameter. The
181 deformability was quantified through the calculation of a deformability index (equation 2)
182 [32]:

183 $$D = 100 - \frac{L_e}{L} \times 100 \quad (2)$$

184 where D is deformability, L_e is size of extruded liposomes (nm), L is size of liposomes (nm)
185 prior to extrusion.

186 *2.2.6 Differential scanning calorimetry of EGCG and EGCG lipid blends*

187 To assess thermal characteristics of materials including melting temperatures, phase
188 transitions and heat capacity changes of liposomes, EGCG and ratios of lipid, surfactant and
189 drug mixtures corresponding to that of the liposome formulation, were analysed in the solid
190 state using a TA Instruments Q200 Thermal Analysis Differential scanning calorimetry
191 (DSC). 3 mg of EGCG was weighed into T-Zero aluminium pans and then hermetically
192 sealed. All experimental runs commenced at an initial temperature of 0°C, purged under
193 nitrogen gas, with a scan rate of 10°C/min to 300°C.

194 *2.2.7 In-vitro EGCG release studies*

195 To assess the impact of inclusion of Tween 20 on EGCG from liposomal formulations, a
196 side—by-side diffusion cell (PermeGear diffusion cell, Hellertown, USA) was maintained at
197 35 °C. Release was assessed over a 24 hour period from an EGCG aqueous solution (0.1
198 mg/mL) and EGCG-loaded liposomes (final loading for liposomes formulated with 0, 2, 6
199 and 10 % w/w Tween 20 was 0.80, 0.55, 0.17 and 0.04 mg/mL respectively). 10 mL of each
200 formulation was placed into the donor side of the diffusion cell and release across a 50 nm
201 membrane (Whatman®) into the receiver side containing 100 mL of dermal dissolution
202 media with a stirrer was measured. The release media was sampled with volume replacement
203 (0.5 mL) over 24 hours and analysed using HPLC-UV quantification.

204 *2.2.8 In-vitro drug release kinetics*

205 Several kinetic drug release mathematical models were used to assess drug release from the
206 formulations. The best-fit to the mathematical models described below confirmed the
207 appropriate release kinetics:

208 *Zero order model*: $\frac{M_t}{M_\infty} = k_0 \cdot \mathrm{t}$ (3)

209 where M_t/M_∞ is the drug fraction released at time t and k_0 is the zero-order release constant.

210 *First order model*: $\frac{M_t}{M_\infty} = 1 - e^{-k_1 t}$ (4)

211 where M_t/M_∞ is the drug fraction released at time t and k_1 is the first-order release constant.

212 *Higuchi model*: $\frac{M_t}{M_\infty} = k_H \cdot t^{\frac{1}{2}}$ (5)

213 where M_t/M_∞ is the drug fraction released at time t and k_H is the Higuchi constant.

214 *Korsmeyer-Peppas Model*: $\frac{C_t}{C} = Kt^n$ (6)

215 where C_t/C is fraction of drug released at time t, k is the release rate constant. The value of n
216 is valuable in understanding drug release mechanisms. When $n \leq 0.45$ drug release is
217 diffusion controlled and can be referred to as 'Fickian' diffusion and when $n > 0.89$ the
218 diffusion is indicative of erosion controlled drug release or class-II kinetics. For situations
219 where $0.45 < n \leq 0.89$ the diffusion is a complex mixture of both processes and often termed
220 anomalous transport. In all cases this is based on the assumption of release from a cylinder
221 and applied to cumulative release rates < 60 % [48]

222 Mathematical models to assess release kinetics were fit using Microsoft Excel®. Zero order,
223 first order, Higuchi and Korsmeyer-Peppas release profiles were applied to release from drug
224 solution and drug loaded liposome solution following which regression analysis techniques
225 were employed to determine the probable drug-release. The release kinetic model displaying
226 the highest r^2 metric (≥0.95) was determined to be the mechanism, by which release occurred.

227 *2.2.9 Liposome stability*

228 The stability of liposomes was determined, as prepared in water, through the assessment of
229 particle size over a 28-day period, stored in a stability cabinet maintained at 25 ± 2 °C
230 (Firlabo, France) at a humidity of 60 % ± 5 %. Mean particle sizes were determined on days

231 1, 2, 7, 14, 21 and 28 by DLS. Furthermore, the encapsulation efficiency of drug loaded
232 liposomes was assessed over 4 weeks as detailed in section 2.2.4.

233 *2.2.10 Development of an in-vitro cellular dermal model*

234 To develop an *in-vitro* system to assess cellular toxicity and cellular uptake of
235 deformable liposomes into representative human dermal tissue, two dermal cell line were
236 examined. Human dermal fibroblasts (HDFa) were cultured in Medium 106 supplemented
237 with low serum growth supplement. Human epidermal keratinocytes (HaCaT) cells were
238 revived and sustained in high glucose supplemented DMEM media. Media was replaced
239 every 3 days. At 70-80% confluency, media was discarded and cells detached using
240 Trypsin/EDTA incubated for 5 min, prior to trypsin neutralisation with 3 mL growth media
241 and subsequent centrifugation at 1200 rpm for 10 min and resuspension in fresh media prior
242 to being utilized for subsequent studies

243 *2.2.11 Cellular toxicity of liposomal formulations towards HDFa and HaCat cells*

244 To determine the cytotoxicity profile of EGCG towards HDFa and HaCat cells, a
245 (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) assay
246 [49] was performed to measure cell viability after exposure to increasing concentrations of
247 EGCG for 24 hours. Cells were seeded at a density of 50 x 10^3 cells per well into a 96-well
248 plate and grown for 3 days. Thereafter, media was removed and cells were exposed to 100 µL
249 of 0.1-100 µM EGCG and incubated for 24 hours at 37°C. Subsequently, 25 µL of a 12.5:1
250 (XTT: menadione) was added each well and incubated for 3 hours at 37°C prior to the
251 absorbance being read at 450 nm. Assessment of EGCG toxicity to these cells was conducted
252 through analysis of changes in XTT absorbance with increasing drug concentration.

253 *2.2.12 Intracellular uptake of deformable liposomes into HDFa and HaCat cells*

254 Liposomes, both deformable and non-deformable, were formulated with the addition of 0.25
255 mL of a 0.1 mg/mL DilC during the lipid mixing stage. Unentrapped DilC was removed by
256 centrifuging liposomes at 18,000 g for 30 min. Coverslips were coated for 30 min with poly-
257 l-lysine (0.01 % w/v) prior to the addition of cells at a density of 50 x 10^3 cells per coverslip.
258 After 24 hours, DilC loaded liposomes were diluted with 1 part of supplemented media (as
259 clarified in materials) and were then added to the coverslips and incubated for 2 hours at
260 37°C.

261 Thereafter, coverslips were washed and fixed with 4 % w/v paraformaldehyde for 5 minutes
262 at room temperature. Subsequently, coverslips were mounted onto glass slides with the
263 addition of a DAPI-containing mounting media. Cover slips were subsequently analysed in
264 an upright confocal microscope (Leica SP5 TCS II MP) and visualised with a 40× oil
265 immersion objective. Images were acquired using a helium-neon laser at 633 nm to visualise
266 DilC and a helium–neon laser to visualise DAPI at 461 nm.

267 *2.2.13 Statistical analysis*

268 Unless otherwise stated, all results are presented as mean ± standard deviation (SD).
269 Replicates of at least 3 were used for all studies. For multiwell plate assays replicates of 6
270 were used for each experimental condition with the study replicated 3 times. A one-way
271 ANOVA was used to determine any statistically significant difference between means tested
272 ($p \leq 0.05$). A post-hoc Tukey's multiple comparisons test was then applied to assess
273 differences between groups. All the calculations were carried out using Graphpad 6
274 (GraphPad Inc., La Jolla, CA).

275 **3. Results and discussion**

276 Emerging treatments for cancer management involve chemoprevention and chemoprotection.
277 Current anticancer agents tend to demonstrate a poor safety profile in addition to possess a
278 wide range of unpleasant side effects [10-12]. However, phytochemical flavonoids, such as
279 EGCG, are increasingly being investigated for their chemoprevention properties [13-15].

280 EGCG is a flavonoid found in green tea that possesses cytotoxic effects in cancerous skin
281 cells and thus may be a potentially viable candidate as a pharmacological anti-cancer agent
282 [16], given that it has been observed to induce apoptosis in cancer cells without affecting
283 normal cells [50, 17], in addition to the modulating expression of a number of genes involved
284 in cell proliferation, cell-cell contact and cell-matrix interactions [51].

285 However, the penetration of drugs across the skin is significantly hindered by the skin's
286 inherent barrier properties [25]. The use of deformable liposomes to aid dermal cellular
287 penetrability and uptake may be advantageous in the targeting of neoplastic agents to deeper
288 skin cellular layers when compared to conventional liposomes which may not be able to
289 penetrate through the narrow pore of the stratum corneum [39].

290 This focus of this study was to develop EGCG loaded slow release deformable liposomes.
291 EGCG liposomes were formulated with PC and cholesterol with the inclusion of Tween 20 as
292 a edge-activator with incorporation of up to 10 % w/w. Liposomal characteristics including
293 liposome size, charge, encapsulation efficiency, DI, release profile, stability, ceulluar toxicity
294 and uptake were was assessed.

295 ***3.1 Liposome characterisation***

296 The impact of the inclusion of Tween 20 within the liposomal formulation on
297 liposome characteristics were observed. As the surfactant loading in the bilayer of 'empty'
298 liposomes increased, liposome diameter decreased from 206 ± 24 nm for liposomes

299 formulated with no surfactant to 102 ± 11 nm for liposomes formulated with 10 % w/w
300 Tween 20 (Figure 1A). As the surfactant loading in the bilayer of EGCG loaded liposomes
301 increased, liposome diameter decreased, from 258 ± 17 nm for liposomes formulated with no
302 surfactant compared with 105 ± 13 nm for liposomes formulated with 10 % w/w Tween 20
303 (Figure 1B). The decrease in size was statistically significant ($p \leq 0.05$) for 'empty' and
304 EGCG loaded liposomes formulated with no surfactant compared with liposomes loaded with
305 2, 6 and 10 % w/w Tween 20.

306 [Figure 1 near here]

307 The inclusion of surfactants into liposome formulations have previously been demonstrated
308 to decrease liposome size when compared to liposomes formulated in the absence of
309 surfactant [26, 32]. This may be as a result of a destabilising effect imparted by the surfactant
310 on the bilayer [52], which results in a greater interaction of the phospholipid bilayer with the
311 aqueous phase. A consequence of this would then be the overall formation of liposomes with
312 a smaller diameter giving a greater surface area in contact with the aqueous phase. The
313 inclusion of surfactant has been previously reported to decrease liposome size in comparison
314 to conventional liposomes. A study formulating liposomes with Phospholipon® 90 G and
315 both Tween 80 and Span 80 reported a size reduction from 207 nm to 139 nm following
316 inclusion of the surfactants [32].

317 A liposome preparation which is homogenous in size is important as final liposome
318 size will partly determine the level of tissue distribution *in-vivo* in addition to influencing
319 drug release kinetics. A polydispersity of up to 0.3 is considered homogenous [53, 32, 54]. As
320 the loading of surfactant increased, the polydispersity of the liposomal formulation did not
321 significantly change for 'empty' liposomes (Figure 1a) whilst a significant decrease was
322 observed for those loaded with EGCG ($P \leq 0.0001$) from 0.3 to 0.2 (Figure 1b). Therefore,

the inclusion of Tween 20 within the liposome formulation appeared to improve homogeneity (although liposome production method may also impact this quality).

The magnitude of the zeta potential (ζ) indicates the degree of electrostatic repulsion between adjacent, similarly charged particles in a dispersion. Thus, it is a fundamental parameter thought to affect stability of liposomal formulations. All formulated liposomes demonstrated a near neutral charge (Table 1). A neutral liposomal surface charge is important to avoid skin irritation [55] however, this may subsequently lead to particle flocculation due to a lack of electrostatic repulsion between liposomes causing them to cluster [56].

[Table 1 near here]

In liposomes formulated with EGCG, as Tween 20 loading increased, a statistical significant decrease in EGCG entrapment was observed, ($P \leq 0.0001$), from 80.0 ± 3.0 % when no surfactant is present to 4.3 ± 3.0 % with a 10 % w/w loading of surfactant (Figure 2). This decrease in EGCG loading may be related to the difference in the molecular weight of EGCG and Tween 20. Tween 20, with larger molecular weight of 1227.54 g/mol compared to that of EGCG (386.65 g/mol), may be assumed to be better poised to displace EGCG from the bilayer. Further, the hydrophobic tail of Tween 20 would have a high affinity to the chains in PC disrupting the liposome structure as well as displacing EGCG from the bilayer [57-59]. Furthermore, Tween 20 is known to enhance the solubility of drugs and therefore, as not all would be entrapped within the bilayer, this may allow increased EGCG solubilisation within the liposomal rehydration media [60]. It is also possible that the coexistence of vesicles and mixed micelles at high surfactant concentrations [61] may have reduced the compound entrapment in mixed micelles.

[Figure 2 near here]

346 The degree of deformability of each formulation was determined by extrusion through
347 a polycarbonate filter with a pore size of 50 nm. The mean particle size and the polydispersity
348 index of liposomes was quantified before and after filtration to assess liposome ability to
349 regain size after having being forced through a pore size smaller than their original diameter.
350 The DI is defined as the degree the liposomes deformed; the greater the degree of
351 deformation the less elastic the liposomes are as they were unable to regain their previous
352 larger size. The DI following extrusion decreased with statistical significance ($P \leq 0.0001$) as
353 surfactant loading increased in 'empty' liposomes, from 71 ± 6 for liposomes formulated with
354 no surfactant compared with 26 ± 3 % for liposomes formulated with 10 % w/w Tween 20
355 respectively. EGCG liposomes formulated with Tween 20 demonstrated a statistically
356 significant decrease ($P \leq 0.0001$) in DI from 74 ± 8 for liposomes formulated with no
357 surfactant compared with 37 ± 7 for liposomes formulated with 10 % w/w Tween 20 (Figure
358 3). These observations imply the liposomes were displaying elastic properties as they could
359 deform in order to pass through an opening smaller than its own diameter whilst, to a certain
360 degree regaining its size. It is important to address the initial size of liposome. Those
361 formulated with Tween 20 were of a smaller diameter thus would require less deformation to
362 fit through the pores. However, the pore size was smaller than liposome diameter in all cases
363 and the DI equation requires the ratio of pre to post extrusion size. Additionally, the presence
364 of EGCG in the liposome formulation did not appear to affect the DI compared with
365 liposomes formulated without. A study formulating liposomes with Phospholipon® 90 G and
366 both Tween 80 and Span 80 saw a size reduction observed surfactant to decrease the DI from
367 51 ± 4 to 17 ± 5 [32].

368 [Figure 3 near here]

Liposomes formulated with surfactant can deform as the surfactant has a propensity for highly curved structures (e.g. micelles and liposomes), thus diminishing the energy required for particle deformation. The surfactant is able to diminish the energy required for particle deformation and accommodate particle shape changes of the liposomes under stress [62]. These surfactants may have interacted with the PC with strong affinity but in reversible mode. The reversible binding mode might have provided the deformability upon physical stress [38].

For liposomes to deform, a source of energy is required [63-65]. In our systems, 'energy' was supplied to this system in the form of pressure as a result of the action of the syringe driver. The larger the concentration of surfactant included within the formulation, the greater the energy the liposome as a whole is be able to retain [65]. It is postulated that this energy is used to reorientate the lipid bilayer structure, and since all systems tend toward the lowest state of free energy, the energy stored in this structure will be expelled once the liposome has passed through the pore and there is no longer any pressure forcing the bilayer to remain in an 'unnatural state' [35, 36, 66]. This energy can then be expended into reforming the liposome. Some energy will be lost during passage as heat or non-plastic deformation, therefore it was not possible to attain a DI of 0 %.

The energy used to alter the bilayer of a liposome containing no surfactant does not benefit from the extra 'storage space' of a surfactant, thus energy may be expended to rupture the membrane causing liposome size to decrease [65]. Despite the potential for excess energy in liposomes formulated with Tween 20, liposomes were not able to fully regain their pre-extrusion size. Some energy will always be lost in the friction of the particles moving through the pores as heat [67]. An increase in surfactant loading may bring the liposomes closer to 100% reformation [65]. Further, liposomes unable to fit through the pores or lipid aggregates

393 from ruptured liposomes may cause blockages. This may lead to an increase in pressure in the
394 vessel causing more turbulence leading to the rupture and non-uniform reformation of
395 liposomes. Additionally, *in-vivo*, liposomes would be expected to move across the skin
396 following an osmotic transepidermal gradient as has been reported in many similar studies
397 concerning the dermal and transdermal delivery of drug [64, 39, 65, 32]. Such lipid carriers
398 are miscible with the epidermal lipids present within the barrier of the stratum corneum thus
399 would be able to penetrate into deeper layers of the skin [68-70]. Furthermore, the skin is
400 warmer than room temp (35 °C compared to 20 °C). Temperature governs the energy term of
401 enthalpy therefore the liposomes would have more energy to be even more flexible and cross
402 the stratum corneum.

403 ***3.2 Differential scanning calorimetry investigations of EGCG and EGCG lipid blends***

404 Differential scanning calorimetry (DSC) has been widely used in understanding the
405 thermal characteristics of materials where an insight into a range of thermal properties
406 including melting temperatures, phase transitions and heat capacity changes can be obtained.
407 It has been observed that drugs with melting point of < 200 °C are better poised to cross the
408 SC [71, 24], therefore observing the effect of formulation parameters on the melting point
409 would aid formulation development. The glass transition temperature (T_c) of EGCG was
410 identified at 220 °C (peak c) and the melting point (T_m) of EGCG was at 245 °C (peak d)
411 (Figure 4) and concurred with those reported by Cho et al (2008) where the T_m of GCG (an
412 epimer of EGCG) was at 223 °C, the T_c of EGCG was at 235 °C and the T_m of EGCG was at
413 246 °C. Cho et al also observed a peak at 97 °C and determined it to be the conversion
414 temperature of EGCG into GCG. Therefore, the first two troughs (peak a and b) observed in
415 the scan may be representative of the epimer GCG [72]. Differences between reported values
416 and those observed here may be due to differences in EGCG sample purity.

417 [Figure 4 near here]

418 The DSC of the lipid (PC and cholesterol) and Tween 20 blend observed the T_m of
419 this mixture to be 172 °C (Figure 5A). There is also a smaller absorption peak at 208°C
420 which would be attributed to one of the liposomal components. Upon addition of EGCG to
421 this mixture, comparison to the aforementioned DSC data observed the melting point shifted
422 to 191 °C (Figure 5B). Furthermore the T_m of EGCG decreased from 242°C to 191°C
423 illustrating that the surfactant loaded liposomes could decrease the T_m of EGCG thus
424 potentially improving partitioning across a membrane [73-75]. Furthermore, the addition of
425 EGCG caused the peak at 208°C to either disappear or shift into the main peak observed at
426 191°C.

427 [Figure 5 near here]

428 ***3.3 EGCG release studies from liposomal formulations***

429 The release of EGCG from solution and liposomal formulations was studied over a 24-hour
430 period (Figure 6). Liposomes appeared to retard the release of EGCG in comparison to
431 release across the membrane from EGCG in solution. Furthermore, with increasing the
432 loading of Tween 20 within liposomal formulations (0 to 10 % w/w), EGCG release
433 increased from 14 ± 1 % at 24 hours for 0 % w/w Tween 20, to 94 ± 5 % at 24 hours for 10 %
434 w/w Tween 20. The cumulative percentage released after 24 hours was significant between
435 the solution and liposomes loaded with 0%, 2%, and 6% w/w of Tween 20 ($P \leq 0.0001$). The
436 inclusion of surfactant enables an increase in drug solubility of poorly soluble compounds
437 thus explaining the increase in drug release at higher loadings of surfactant. Such properties
438 are already exploited to improve the oral delivery release profiles of poorly soluble
439 compounds in self-emulsifying drug delivery systems with four drug products [76, 77],
440 Sandimmune® and Sandimmun Neoral® (cyclosporin A), Norvir® (ritonavir), and

441 Fortovase® (saquinavir) on the pharmaceutical market [76]. It is worth noting that as
442 surfactant loading increased, EGCG entrapment decreased thus a lower concentration
443 gradient would be observed. This did not appear to retard EGCG release.

444 [Figure 6 near here]

445 An increased rate of release was observed from the EGCG solution compared with
446 liposome formulations over the 24 hours observed (Table 2). Further, as the loading of
447 surfactant increased, the rate of EGCG release increased (from 0.034 ± 0.013 to 0.993 ±
448 1.013 for liposomes loaded with 0 % and 10 % of Tween 20 respectively based on the
449 Korsmeyer-Peppas model). Thus, surfactant appears to increase drug release/encourage
450 liposome breakdown, particularly at 10 % w/w where the rate was 10 fold greater than that at
451 6% w/w. The surfactant would increase drug solubility thus explaining why an increase in
452 drug release is observed at higher loadings of surfactant. The inclusion of surfactant
453 destabilizes the vesicle bilayers by reducing the amount of work required to expand the
454 interface allowing the liposome to become more flexible [40, 38, 39] and move through the
455 membrane. Additionally, it has been suggested that the mechanism of the *in-vitro* release
456 seems to be the formation of transient pores in the lipid bilayer, through which drugs are
457 released to the extra-liposomal medium (Wang, Wang et al. 2016).

458 [Table 2 near here]

459 Based on the values of the determination coefficient (R^2), as well as AIC values
460 (Akaike Information Criterion), the model that best describes EGCG release from all
461 liposomal formulations is Korsmeyer-Peppas model (highest R^2 and lowest AIC). The
462 diffusion release exponent value revealed a range of release mechanisms for each formulation.
463 Liposomes formulated with 0%, 6 % and 10% w/w Tween 20 had an exponent value of 0.839
464 ± 0.072, 0.836 ± 0.116 and 0.722 ± 0.247 respectively indicating the release is a complex

465 mixture of the diffusion (flux due to molecular diffusion and the concentration gradient) and
466 erosion controlled drug release or class-II kinetics (diffusion not based on concentration
467 gradient) processes and often termed anomalous transport [78]. Liposomes formulated with 2
468 % w/w Tween 20 observed an exponent value of 0.913 ± 0.186 indicative of erosion
469 controlled drug release or class-II kinetics [48].

470 ***3.4 Stability of EGCG loaded deformable liposomes***

471 The impact of long-term storage of EGCG-loaded liposomes formulated with 2 %
472 w/w Tween 20 was assessed during storage in stability cabinets maintained at 25 ± 2 °C
473 (Firlabo, France) at a humidity of 60 % ± 5 %. Liposomes formulated with 2% w/w Tween
474 were selected in this study as it had the highest EGCG entrapment compared with the higher
475 loadings of surfactant thus will be taken forward for cell uptake studies. The impact of this
476 storage on size (Figure 7) and encapsulation efficiency (Figure 8) was assessed. EGCG
477 loaded liposomes formulated with and without surfactant maintained a consistent size over
478 time (Figure 7) with no statistically significant difference in size during the storage period.
479 Previous reports have highlighted that aggregation is common upon liposomal formulation
480 storage, and results in vesicle size growth [79] particularly, though not always, with neutral
481 liposomes [56]. However, the inclusion of Tween 20 into the deformable liposomes may have
482 prevented this phenomenon and may be a result of surfactant destabilising the lipid bilayer
483 and reducing the energy required to expand the interface, thus allowing maintenance of
484 smaller structures. It appears the inclusion of surfactant prevents this phenomenon which
485 correlates with similar studies [80].

486 [Figure 7 and 8 near here]

487 Furthermore, encapsulation efficiency showed a trend of decreasing loading from 43 ± 7 % to
488 42 ± 12 %, 39 ± 9 %, 30 ± 11 % to 30 ± 6 %, for liposomes formulated with 2 % w/w Tween

489 20 (Figure 8). Tween 20 is able to increase compound solubility, therefore, this may allow
490 EGCG to solubilise within the liposomal media [60] and leach out over time.

491 ***3.5 Cellular toxicity of liposomal formulation towards HDFa and HaCat cells***

492 Whilst topical formulations are applied directly into the skin, various connective layers
493 making up the skin are important for drug delivery. The skin primarily consists of the
494 epidermis, dermis and subcutaneous layers and each layer has a unique combination of cells,
495 connective tissue, components and functions. Skin cancers develop in the upper layers of the
496 skin spanning the dermal and epidermal layer, and any formulation system should consider
497 the impact of formulation systems on these tissue layers for the delivery of drugs.

498 In order to assess cellular toxicity of EGCG to these cells, we adopted two *in-vitro* cell
499 culture systems, namely human keratinocyte and human fibroblast cells. To determine the
500 cellular viability cytotoxicity of EGCG towards HDFa and HaCat cells, an XTT assay was
501 performed to measure cell death after exposure of cells to different concentrations of drug for
502 24 hours (Figure 9).

503 [Figure 9 near here]

504 As the concentration of EGCG was increased from 0.1 to 100 μM, HDFa cell viability
505 decreased (Figure 9A) with statistical significance ($P \leq 0.0001$). This may be due to toxicity
506 or death of damaged cells in which EGCG induced apoptosis [81, 82]. Whilst limited data
507 exists on the cytotoxicity of EGCG towards dermal tissues, a study observing growth
508 inhibition in multiple cell lines, observed that EGCG at 40 μM had little or no inhibitory
509 effect on the growth of WI38 cells, normal human fibroblast cells [83]. Cell viability was
510 maintained across the concentration range of 0.1–100 μM on HaCat cells (Figure 9B). No
511 statistically significant difference was observed in cell viability. Furthermore, EGCG has

512 been reported to impart protective effects in HaCat cells exposed to external stressors
513 including UVA and UVB radiation [84, 85]. Whilst some of our formulations exceeded this
514 concentration of EGCG as a whole, the retarded release profile of the liposomes would be
515 expected to result in an overall lower temporal concentration profile exposure to these cells,
516 significantly below 100 μM.

517 ***3.6 Cellular liposomal uptake assay into HDFa and HaCat cells***

518 A primary goal for our studies was to demonstrate uptake of deformable liposomes
519 loaded with EGCG into a cell culture skin model. EGCG loaded liposomes were incubated
520 with both HaCat (Figure 10) and HDFa (Figure 11) cells to assess the cellular uptake of these
521 formulations. Liposomes formulated with 2% w/w Tween 20 were selected, a result of the
522 highest EGCG entrapment compared with the other surfactant loadings. Fluorescently
523 labelled liposomes loaded with EGCG incubated for 2-hours with both HaCat and HDFa cells
524 seeded onto collagen-coated coverslips and the cellular localisation of these liposomes was
525 determined using confocal microscopy. Following a 2-hour incubation with the cells,
526 intracellular localisation of labelled liposomes were clearly evident, confirming the
527 successful uptake into both HaCat and HDFa cells.

528 [Figure 10 and 11 near here]

529 Extraneous particle cell uptake is dependent upon influences such as particle size,
530 charge, affinity etc. [86-88]. There are four proposed methods of liposome uptake into cells:
531 stable adsorption, endocytosis, fusion of the lipid bilayer with the cell plasma membrane and
532 lipid transfer [43, 89]. It is unclear which of these, or another process, occurred in this study,
533 however, these methods of uptake are not mutually exclusive and any combination may occur
534 in a given experimental circumstance [43]. The interaction of nanoparticles with cell
535 membrane seems to be most affected by particle surface charge. The cell membrane surface

536 is dominated by negatively charged sulphated proteoglycans molecules (vital in cellular
537 proliferation and migration) [90, 91]. These molecules are associated with
538 glycosaminoglycan side chains (heparan, dermatan, keratan or chondrotine sulfates) which
539 are anionic, and interaction between proteoglycans and liposomes, if positively charged, tend
540 to be largely ionic [92]. The liposomes applied to the cells in this study had a ζ of 4 ± 1
541 suggesting an ionic interaction may have occurred. A study applying cationic liposomes
542 formulated with the cationic lipids Lipofectin, Tfx-50, and Lipofectamine in oligonucleotide
543 delivery to HaCat cells observed liposome uptake within 24 hours [93]. Furthermore,
544 research developing chemotherapy against malignant melanoma using mouse B16 melanoma
545 cells as well as Normal Human Dermal Fibroblasts observed a greater uptake of cationic
546 liposomes by cells in the injection site compared with neutral liposomes due to the
547 electrostatic interaction with the negative-charged phospholipid membrane of cells [94].

548 It should be noted that the confocal microscopy studies demonstrated the possibility of the
549 delivery of deformable liposomes to relevant dermal tissues using *in-vitro* cell culture
550 techniques. However, the application of such formulations could also be assessed using *ex-*
551 *vivo* human or animal dermal tissues. The ultimate aim of this delivery system was to
552 improve dermal cell uptake and delivery a controlled release of active agent, thus from a
553 regulatory perspective, pharmacokinetic data is not required as drug is not intended to reach
554 the blood stream [95].

555 This study did not address skin penetration. The liposomes formulation recommended to take
556 into further studies would be that loaded with 2% w/w Tween 20. In order to ascertain the
557 extent of carrier and drug permeation a skin strip test may be appropriate [70]. This involves
558 the use of an adhesive tape to strip the skin layer by layer and quantifying lipid and drug on
559 each layer[96]. Further, whilst the most appropriate animal model for human skin is the

560 porcine skin tissue, sample-to-sample variability in addition to differences in the lipid dermal
561 matrices often results in an altered permeability profile limiting the wider human translational
562 goals [97-99]. The use of skin tissue samples would also help determine whether intact
563 liposomes deliver EGCG to dermal cells or if EGCG delivery is as a result of liposomes
564 being mixed with lipids present in the stratum corneum.

565 **4. Conclusion**

566 Skin cancer is emerging as an increasing public health problem particularly in
567 developed countries. Current treatments include surgery to remove the tumour as well as
568 topical formulations. Such treatments may not be suitable for all patients as they are
569 associated with an unpleasant aesthetic profile as well as side effects. A nanoparticle delivery
570 system such as deformable liposomes applied topically for the direct dermal delivery of
571 compounds would be valuable in carrying compounds across the stratum corneum at a
572 controlled rate whilst limiting side effects. The use of naturally occurring compounds such as
573 EGCG have been found to be successful as chemopreventative and chemoprotective agents.
574 However, formulation of such compounds has been limited in success due to a limited
575 bioavailability of promising agents and inefficient delivery systems. We developed a novel
576 deformable liposome formulation loaded with EGCG and systemically investigated the
577 loading, uptake and *in-vitro* release of EGCG from these nanoparticles. This study has found
578 deformable liposomes could be valuable in targeted delivery whilst offering controlled
579 release of the compound [13, 100]. We have demonstrated that as the amount of Tween 20 in
580 the liposomal bilayer is increased, liposome size decreased and elasticity increased. As the
581 loading of Tween 20 in the liposome was increased the EGCG encapsulation decreased. This
582 may have been due to Tween 20 competing for space within the bilayer or due to Tween 20
583 increasing the solubilisation capacity of EGCG. Additionally EGCG release from liposomes

584 found that the liposomes were able to modify the release of drug with complete release
585 observed within 24 hours. Further, our studies demonstrated these liposomes were capable of
586 uptake into epidermal keratinocytes and dermal fibroblasts within 2 hours. This present study
587 demonstrates liposomes formulated with Tween 20 are useful in the development of a
588 controlled release topical formulation for dermal delivery crucial in improving patient
589 compliance thus skin cancer treatment outcomes.

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847

848 **Table 1:** Zeta potential of liposomal formulations formulated in the absence and presence of

849 up to 10% w/w of Tween 20

Surfactant loading (% w/w)	**Zeta potential (mV)**	
	‘empty’ liposomes	EGCG loaded liposomes
0	5.03 ± 1.03	2.41 ± 1.08
2	4.67 ± 1.08	3. 67 ± 0.91
6	3.71 ± 0.90	-0.99 ± 1.01
10	-2.79 ± 0.20	-1.90 ± 0.88

850 Results are presented as the mean ± standard deviation (n=3)

851

852 **Table 2*:*** *In-vitro* SA release kinetics models

Kinetic model	**Parameter**	**Tween 20 Loading (% w/w)_**			
		0	2	6	10
0	$(k_0) \times 10^{-2}$ mg • min^{-1}	1.01 ± 0.05	1.18 ± 0.01	2.75 ± 0.05	7.77 ± 0.02
	R^2	0.95 ± 0.02	0.99 ± 0.01	0.93 ± 0.07	0.72 ± 0.30
	AIC	28.59 ± 4.44	18.82 ± 2.96	57.24 ± 14.21	98.21 ± 25.32
1st	$(k_1) \times 10^{-4}$ min^{-1}	1.07 ± 0.05	1.27 ± 0.13	3.32 ± 0.58	15.04 ± 3.11
	R^2	0.96 ± 0.02	0.986 ± 0.01	0.96 ± 0.04	0.94 ± 0.06
	AIC	25.68 ± 4.40	18.132 ± 6.83	54.03 ± 6.46	4.56 ± 17.86
Higuchi	k_H	0.26 ± 0.01	0.294 ± 0.03	0.71 ± 0.09	2.12 ± 0.14
	R^2	0.83 ± 0.04	0.7513 ± 0.03	0.81 ± 0.05	0.86 ± 0.10
	AIC	47.40 ± 5.92	58.783 ± 4.90	77.32 ± 9.63	99.05 ± 13.13
Korsmeyer-Peppas	k_{KP}	0.03 ± 0.01	0.059 ± 0.08	0.09 ± 0.06	0.99 ± 1.01
	N	0.84 ± 0.07	0.913 ± 0.19	0.84 ± 0.12	0.72 ± 0.25
	R^2	**0.99 ± 0.01**	**0.991 ± 0.01**	**0.99 ± 0.01**	**0.96 ± 0.03**
	AIC	11.26 ± 3.00	27.163 ± 22.24	49.82 ± 8.49	82.76 ± 9.81

853 R^2, coefficient of determination; AIC, Akaike Information Criterion; F is the fraction of drug released at time t; k_0 is the zero-order release
854 constant; k_1 is the first-order release constant; k_H is the Higuchi release constant; k_{KP} is the release constant incorporating structural and
855 geometric characteristics of the drug- dosage form; n is diffusion release exponent.

List of figures

Fig. 1 Liposome size distribution and polydispersity of 'empty' and EGCG loaded liposomes

Liposome size distribution and polydispersity, determined by DLS, comparing (A) 'empty' and (B) EGCG loaded formulations with Tween 20 (0-10 % w/w). Liposomes were prepared by the dry film hydration method and EGCG added during the lipid mixing stage. Data represents mean ± SD. n=3 independent batches. **** indicates statistical comparison between the size of liposome formulations with a $P \leq 0.0001$. # # indicates statistical comparison between the polydispersity of liposome formulations with a $P \leq 0.01$.

Fig. 2 Entrapment efficiency of EGCG in liposomes formulated with 0-10% w/w Tween 20

Entrapment efficiency (%) of EGCG in liposomes formulated with varying amounts of Tween 20 (0-10% w/w) Data represents mean ± SD. n=3 independent batches. **** indicates statistical comparison between the entrapment efficiency of liposome formulations with a $P \leq 0.0001$.

Fig. 3 Deformability index for 'empty' and EGCG loaded liposomes

Deformability index following extrusion through 50 nm membranes for 'empty' and EGCG loaded liposomes with increasing surfactant loading up to a maximum of 10% w/w. Liposomes were prepared adapting the dry film method adding the surfactant and adding EGCG during the lipid mixing stage. The preparation was vortexed and then extruded though the membranes. Data represents mean ± SD. n=3 independent batches. **** indicates statistical comparison between the DI of liposome formulations with a $P \leq 0.0001$.

Fig. 4 Differential scanning calorimetry scan of EGCG

All experimental runs commenced at an initial temperature of 0 °C with a scan rate of 10 °C/min to 300 °C. Peak a and b are related to the epimer of EGCG, GCG. Peak c represents the glass transition temperature (T_c) of EGCG was at 220 °C and the melting point (T_m) of EGCG was at 245 °C.

Fig. 5 Differential scanning calorimetry analysis scans of PC, cholesterol and Tween 20 and EGCG blends

DSC analysis scans of (A) PC, cholesterol and Tween 20 blend and (B) PC, cholesterol, Tween 20 and EGCG blend. The T_m of the lipid mixture is 172 °C, and upon addition of EGCG, the T_m was 191 °C. All experimental runs started at an initial temperature of 0 °C, purged under nitrogen gas, with a scan rate of 10 °C/min to 300 °C.

Fig. 6 *In-vitro* percentage EGCG cumulative release profiles from solution and liposomal formulations

EGCG release profiles from solution and liposomes formulated with 0, 2, 6 or 10 % w/w Tween 20 over 24 hours. Liposomes were prepared adapting the dry film method adding the surfactant and EGCG during the lipid mixing stage. A diffusion cell dialysis system was used to evaluate *in-vitro* drug release. Data represents mean ± SD. n=3 independent batches. ****

893 indicates statistical comparison between the EGCG release of liposome formulations with a P
894 ≤ 0.0001.

895 **Fig. 7** Stability of EGCG loaded liposomes as determined by size

896 Size of EGCG loaded liposomes formulated with 0-10% w/w Tween 20, using DLS,
897 formulated with up to 10% w/w Tween 20 measured on various days (1, 7, 14, 21 and 28).
898 Data represents mean ± SD. n=6 independent batches.

899 **Fig. 8** Liposome encapsulation efficiency for EGCG

900 Liposome encapsulation efficiency for EGCG in liposomes formulated with 2 % w/w Tween
901 20 liposomes over 28 days. Liposomes were prepared adapting the dry film method adding
902 the surfactant and drug during the lipid mixing stage. The preparation was then washed via
903 centrifugation. The quantity of EGCG in supernatant over 28 days was then analysed by
904 HPLC coupled with UV detection to assess liposome stability. Data represents mean ± SD.
905 n=6 independent batches.

906 **Fig. 9** Cellular toxicity of EGCG

907 HDFa (A) and HaCat (B) cells were grown on a 96-well plate at a density of 50 x 10^3 cells
908 per well and exposed to various concentrations of EGCG (0.01-100 µM) for 24 hours.
909 Thereafter 25 µL of a 12.5:1 parts mixture of XTT to menadione was added each well. Plates
910 were incubated for 3 hours at 37°C and the absorbance read at 450 nm. Data is reported as
911 mean ±SD with 6 replicates per compound in at 3 independent experiments. ****, ***, **, *
912 indicates statistical comparison between the entrapment efficiency of liposome formulations
913 with a P ≤ 0.0001, 0.001, 0.01 and 0.05 respectively.

914 **Fig. 10** Localisation of DilC labelled liposomes loaded with EGCG and 2% w/w Tween 20 in
915 HaCat cells

916 Cells were grown on the coverslips for 2 days. Cell nuclei were visualised using (A) DAPI
917 (Blue). Liposomes were formulated with DilC for visualisation (B) (yellow). Liposome
918 localisation within the cell is shown in the merged image (C).

919 **Fig. 11** Localisation of DilC labelled liposomes loaded with EGCG and 2% w/w Tween 20 in
920 HDFa cells

921 Cells were grown on the coverslips for 2 days. Cell nuclei were visualised using (A) DAPI
922 (Blue). Liposomes were formulated with DilC for visualisation (B) (yellow). Liposome
923 localisation within the cell is shown in the merged image (C).

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