The effect of lipid composition and liposome size on the release properties of liposomes-in-hydrogel

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Abstract

To study the release of liposome-associated drugs into hydrogels, we designed and synthesized two pH-sensitive rhodamine derivatives to use as model compounds of different lipophilicities. The dyes were fluorescent when in the free form released from liposomes into the chitosan hydrogel, but not when incorporated within liposomes. The effect of liposomal composition, surface charge and vesicle size on the release of those incorporated dyes was evaluated. The lipophilicity of the rhodamine derivatives affected both the amount and rate of release. While liposome size had only a minor effect on the release of dyes into the hydrogel, the surface charge affected the release to a greater extent. By optimizing the characteristics of liposomes we could develop a liposomes-in-hydrogel system for application in wound therapy. We further characterized liposomes-in-hydrogel for their rheological properties, textures and moisture handling, as well as their potential to achieve a controlled release of the dye. The polymer-dependent changes in the hydrogel properties were observed upon addition of liposomes. The charged liposomes exhibited stronger effects on the textures of the chitosan hydrogels than the neutral ones. In respect to the ability of the system to handle wound exudates, chitosan-based hydrogels were found to be superior to Carbopol-based hydrogels.

Key words: liposomes; hydrogels; *in vitro* release; chitosan; skin therapy

1. Introduction

A major aim in the development of modern hydrogel formulations such as those currently used in wound dressings, is to achieve the effective and accurate delivery of the required therapeutic agents included in the formulation over a prolonged period of time (Boateng et al., 2008). Among hydrogels, chitosan hydrogels are one of the most studied systems, particularly with respect to their bioadhesiveness. Chitosan has frequently been studied as a possible wound dressing and as a delivery system for therapeutic agents. This is primarily due to its confirmed biocompatible, biodegradable, non-toxic and bacteriostatic properties, as well as its ability to promote wound healing (Denis et al., 2012). While a lot of research on the potential use of chitosan as a wound dressing has focused on plain chitosan hydrogels, chitosan-based hydrogels (Alsarra, 2009; Bhattarai et al., 2010; Ribeiro et al., 2009), chitosan films (Aoyagi et al., 2007; Noel et al., 2008) and other chitosan-based formulations (Salam et al., 2010), relatively little has been published about liposomal chitosan hydrogels.

The rationale behind using liposomes-in-hydrogel as a delivery system is to assure sustained drug release during their prolonged presence at the administration site (Ruel-Gariepy et al., 2002). The release of drugs from drugs-in-liposomes-in-hydrogel systems is affected by different factors related to the physicochemical properties of the drug. The release of amphiphilic/lipophilic drugs, which are assumed to have the ability to penetrate the liposomal membrane, will be determined by the lipid concentration of liposomes added into the gel (Mourtas et al., 2008b).

In the current study we aimed to gain a better insight into the interactions between drug molecules, liposomes and hydrogels. However, the complexity of the liposomes-in-hydrogel delivery system limits a real-time analytical evaluation of drug release from liposomes, which function as a drug reservoir within the hydrogel, delivering the drug to the administration site. For this purpose, pH-sensitive rhodamine compounds of two different lipophilicities were

designed and synthesized to follow their release from liposomes into the hydrogel. The dyes were incorporated in liposomes which varied in lipid composition, surface charge and size.

The use of hydrogels as vehicles provides the required rheological properties required for the incorporated liposomes (Cohen et al., 2012; Mourtas et al., 2007; Mourtas et al., 2008b; Paavola et al., 2000; Pavelic et al., 2001). In addition, the high viscosity of hydrogels acts as a protective mechanism which can stabilize liposomes, as has been previously shown by Mourtas and colleagues (Mourtas et al., 2008b).

An additional important characteristic that makes hydrogels interesting for wound therapy is their bioadhesiveness. The rheological and bioadhesive properties of hydrogel formulations determine their retention time at the administration site and can therefore influence the therapeutic outcome of the treatment. Previously, we have shown the superior bioadhesiveness of chitosan-based liposomal hydrogels as compared to Carbopol-based hydrogels (Hurler and Škalko-Basnet, 2012). However, in the case of wound treatment the bioadhesiveness can be affected by the wound's exudate. Some wounds, such as burns, produce a lot of exudate, which can lead to maceration of the wound bed, whereas other wounds are dry and need additional moisture from the wound dressing for their proper healing (Fulton et al., 2012). Therefore, in this study we also tested the fluid handling properties of both chitosan- and Carbopol-based hydrogels.

2. Materials and Methods

The rhodamine derivatives used in this study, namely MP-4 and MTJ-12 (log p 4.17 and log p 2.32, respectively, as calculated by ChemBioDraw 12.0, CambridgeSoft) were synthesized at the Faculty of Pharmacy, University of Ljubljana, Slovenia (*manuscript in*

preparation). Lipoid S100 (PC, soya phosphatidylcholine >94%) and Lipoid E PG-Na (PG, egg phosphatidylglycerol sodium) were a generous gift from Lipoid GmbH (Ludwigshafen, Germany). Octadecylamine (SA) and high Mw chitosan (Brookfield viscosity 800.000 cps, DD of 77) were a product of Sigma Aldrich Chemistry (St. Luis, USA). Carbopol® Ultrez 10 was obtained from Noveon (Cleveland, USA). Triethylamine was purchased from Merck Schuchardt (Hohenbrunn, Germany) and glycerol was obtained from Merck KGaA (Darmstadt, Germany). All other chemicals used in experiments were of analytical grade.

2.1. Rhodamine derivatives

All ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III NMR instrument operating at 400 MHz and 100 MHz (^{13}C). IR spectra were recorded on a Perkin-Elmer FTIR 1600 spectrometer. Mass spectra were obtained with a Q-Tof Premier mass spectrometer (Centre for Mass Spectrometry, Institute Jožef Stefan, Ljubljana, Slovenia).

3',6'-bis(ethylamino)-2-(3-hydroxypropyl)-2',7'-dimethylspiro[isoindoline-1,9'-xanthen]-3-one

MP-4

^{1}H-NMR (DMSO-d_6, 400 MHz): δ 1.15-1.19 (m, 2H, N-CH_2-C$\underline{H}_2$-CH_2-OH), 1.22 (t, 6H, *J* = 7.25 Hz, 2 × -CH_2-C$\underline{H}_3$), 1.87 (s, 6H, 2 × Ar-CH_3), 3.02 (t, 2H, *J* = 7.51 Hz, N-C$\underline{H}_2$-CH_2-CH_2-OH), 3.10-3.16 (m, 6H, N-CH_2-CH_2-C$\underline{H}_2$-OH, 2 × -C$\underline{H}_2$-CH_3), 4.33 (bs, 1H, -OH), 5.07 (t, 2H, *J* = 5.32 Hz, 2 × -NH-), 6.08 (s, 2H, $H^{4'}$-Ar, $H^{5'}$-Ar), 6.27 (s, 2H, $H^{1'}$-Ar, $H^{8'}$-Ar), 6.96-6.98 (m, 1H, H^{7}-Ar), 7.48-7.50 (m, 2H, H^{5}-Ar, H^{6}-Ar), 7.77-7.79 (m, 1H, H^{4}-Ar) ppm. ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 14.15, 17.02, 31.02, 37.27, 37.47, 54,91, 64.28, 95.61, 104.66, 118.16, 122,19, 123,51, 127.51, 128.15, 130.49, 132.52, 147.58, 150.96, 153.64, 166.93 ppm. IR (KBr) 3425, 3337, 2961, 2858, 1682, 1636, 1620, 1517, 1470, 1421, 1326,

1271, 1219, 1159, 1144, 1042, 1014, 868, 814, 782, 746 cm^{-1}. MS (ESI) *m/z* (rel intensity) 472 (MH^+, 100); HRMS (ESI): Calcd for $C_{29}H_{34}N_3O_3$ $[M+H]^+$ 472.2600, found 472.2597.

3',6'-bis(ethylamino)-2',7'-dimethyl-2-(2-(((2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)ethyl)spiro[isoindoline-1,9'-xanthen]-3-one

MTJ-12

^{1}H-NMR (DMSO-d_6, 400 MHz): δ 0.95 (t, 3H, *J* = 6.90 Hz, -CH_2-$C\underline{H}_3$), 1.22 (t, 3H, *J* = 7.16 Hz, -CH_2-$C\underline{H}_3$), 1.88 (s, 3H, Ar-CH_3), 2.05 (s, 3H, Ar-CH_3), 2.91-3.28 (m, 12H, N-$C\underline{H}_2$-$C\underline{H}_2$-O-, 2 × -$C\underline{H}_2$-CH_3, H^2-G, H^3-G, H^4-G, H^5-G), 3.50-3.53 (m, 1H, H^{6a}-G), 3.74-3.79 (m, 1H, H^{6b}-G), 4.06-4.15 (m, 1H, -OH), 4.54-4.58 (m, 2H, 2 × -OH), 4.65 (bs, 1H, -OH), 4.89 (d, 1H, *J* = 4.9 Hz, H^1-G), 4.93 (t, 1H, *J* = 4.0 Hz, -NH-), 5.18 (t, 1H, *J* = 5.14 Hz, -NH-), 6.12 (s, 1H, $H^{4'}$-Ar), 6.29 (s, 2H, $H^{1'}$-Ar, $H^{8'}$-Ar), 7.01-7.04 (m, 1H, H^7-Ar), 7.31 (d, 1H, *J* = 5.37 Hz, $H^{5'}$-Ar), 7.50-7.52 (m, 2H, H^5-Ar, H^6-Ar), 7.81-7.79 (m, 1H, H^4-Ar) ppm. ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 14.18, 14.27, 17.05, 17.78, 37.53, 42.05, 48.64, 58.06, 61.64, 64.01, 70.40, 70.56, 78.13, 78.35, 92.97, 93.04, 95.59, 104.09, 114.01, 114.17, 118.64, 122.50, 123.73, 127.53, 128.29, 128.51, 130.23, 132.90, 147.93, 148.82, 148.85, 149.40, 149.47, 150.81, 153.23, 167.05. ppm. IR (KBr) 3422, 2926, 1670, 1522, 1495, 1400, 1270, 1201, 1076, 1016, 888, 747 cm^{-1}. MS (ESI) *m/z* (relative intensity) 620 (MH^+, 100); HRMS (ESI): Calculated for $C_{34}H_{42}N_3O_8$ $[M+H]^+$ 620.2972, found 620.2971.

2.2. *Preparation and characterization of liposomes*

Liposomes were prepared by the dry film method. Three different lipid compositions were used for the preparation: namely PC, PC/PG (1/9, molar ratio), and PC/SA (9/3, molar ratio) (Pavelic et al., 2005). The empty liposomes were used for the rheological and textural studies. In brief, the lipid components (26 mmol/L) were dissolved in methanol and the solvent later

removed by evaporation on a rotary vacuum evaporator (Büchi R-124, Büchi Labortechnik, Flawil, Switzerland). The lipid film was rehydrated in 10 ml of distilled water (pH 6.7) and hand-shaken for 10 min. The liposome suspension was kept in a refrigerator overnight before the size reduction and further characterization.

Liposomes containing dyes were prepared in the same manner. Namely, the lipid components were dissolved in methanol and rhodamine dye was added in the organic solution (2 μmol/L). The rhodamine dyes, MP-4 and MTJ-12 (Figure 1) served as the model fluorescent compounds and were especially synthesized to have the targeted lipophilicity. The dyes were designed to be fluorescent only at a pH value of 4 while being non-fluorescent at pH values higher than 6. The solvent was removed by evaporation and the lipid/compound film rehydrated by 10 ml of phosphate buffer (pH 7.4) and hand-shaken for 10 min prior to storage at 4 °C overnight. To remove unentrapped rhodamine dye the liposomal suspension was ultracentrifuged (80000 g, 30 min, Sorvall® WX 100, Thermo Scientific, Waltham, Massachusetts, USA) and the pellet resuspended in 10 ml of distilled water (pH 6.7).

Liposomes of various sizes were prepared by the probe sonication; the liposomal suspensions were cooled in an ice bath and sonicated three times at continuous cycle for 20 s at 40% amplitude by a Cole Parmer Ultrasonic Processor 500 W (Cole Parmer Instruments, Vernon Hills, Illinois, USA).

All liposomal suspensions were characterized for size by dynamic light scattering and zeta potential with a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK).

2.3. Preparation of hydrogels

Hydrogels were prepared as described earlier (Hurler et al., 2012b). In brief, Carbopol hydrogels were prepared by blending of Carbopol Ultrez 10 powder in distilled water (0.5 % w/w, respectively) and adding triethylamine for neutralization. The amount of triethylamine

was adjusted to obtain hydrogels with a pH value of 7. The gels were allowed to swell at room temperature for 24 h before further experiments.

Chitosan hydrogels were prepared as previously described (Hurler et al., 2012b). In brief, high molecular weight chitosan, 2.5 % (w/w), was manually mixed into a blend of acetic acid (2.5 %, w/w) and glycerol (10 %, w/w). The plain chitosan hydrogel (control, not containing glycerol) was prepared in the same manner as chitosan hydrogels containing glycerol and liposomes. The hydrogels were allowed to swell for at least 48 h at room temperature before further use.

2.4. Preparation of liposomes-in-hydrogels

Hydrogels were prepared as described in 2.3. After the swelling time, 10% (w/w) the liposomal dispersion was added and stirred carefully by hand until an even distribution within the hydrogel was achieved (Hurler et al., 2012b).

2.5. Release of rhodamine dyes from liposomes into hydrogel in dye-in-liposome-in chitosan hydrogel system

Liposomes-in-hydrogels made of chitosan were prepared as described in 2.4. The liposomes contained either MP-4 or MTJ-12 rhodamine dyes. All chitosan hydrogels had a pH value of 4. As the liposomes were prepared with a buffer of pH 7.4, the rhodamine dyes within the liposomes were not fluorescent. However, when the incorporated dyes started to diffuse out of the liposomes into the hydrogel vehicle, the rhodamine compounds became fluorescent and thus detectable as fluorescence within the hydrogels. The release of rhodamine compounds was determined at different time intervals (15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 180, 210, 240, 270 and 300 min) fluorimetrically using a Tecan plate reader, Safire² (excitation

wavelength 520 nm for MP-4, 514 nm for MTJ-12, emission wavelength 560 nm for MP-4, 554 nm for MTJ-12). The measured fluorescence activities were normalized.

The list of preparations evaluated for their respective dye release is given in Table 1 (without empty liposomes).

All measurements were performed in triplicate.

2.6. Rheological evaluation of hydrogels

The Carbopol and chitosan hydrogels, both those which were empty and those containing incorporated PC liposomes, were characterized with regard to their rheological properties using a CS-rheometer (RheoStress RS 100 1 Ncm, Peltier TC 81, Haake, Germany). A cone/plate C 35/1° (0.05 mm) measurement system was used at 20 °C in all experiments (Pavelic et al., 2001).

2.7. Texture analysis of chitosan hydrogels

Texture analysis of the hydrogels was carried out at room temperature (TA-XT plus Texture Analyser, Stable Micro Systems Ltd., Surrey, UK) as previously described (Hurler et al., 2012b). In brief, 50 g of formulation were filled into a standard beaker. A disk (40 mm in diameter) was placed 5 mm under the gel surface and then pushed into the gel (10 mm at a speed of 4 mm/s, respectively) and redrawn again. Gel hardness was determined from the resulting force-time plot, and cohesiveness and adhesiveness of the hydrogels were calculated.

Each sample was measured five times. Experiments were performed in triplicate.

206 *2.8. Fluid affinity testing of hydrogels*

207 The Carbopol and chitosan hydrogels, both the empty hydrogels and liposomes-in-hydrogels,
208 were tested with respect to their potential to handle wound exudates. The standard test we
209 used for this purpose is described in the European norm, "BS EN 13726-1:2002 Test methods
210 for primary wound dressings. Part 1 Aspects of absorbency, Section 3.4, Fluid affinity of
211 amorphous hydrogel wound dressings" (Thomas et al., 2005).

212 The gelatin (35 %, w/w) which was selected to mimic a dry wound was prepared in Solution
213 A (salt solution of sodium/calcium chloride containing 142 mmol/L of sodium ions and 2.5
214 mmol/L of calcium ions). The concentrations of ions were adjusted to be comparable to those
215 present in serum and wound fluid (BS EN 13726-1:2002).

216 The swollen gelatin (10 ± 0.1 g) was filled into the barrels of 60 ml syringes, after removing
217 the tip-end of the syringes and then closing this end with a rubber plug to generate a flat
218 surface. Hydrogel (10 ± 0.1 g), namely the empty Carbopol hydrogel, liposomes-in-Carbopol
219 hydrogel, empty chitosan hydrogel or liposomes-in-chitosan hydrogel, were filled onto the top
220 of the stiffened gelatin plug. After incubation for 48 h ± 30 min at 25 ± 2 °C, the hydrogels
221 were removed gently from the gelatin and re-weighed.

222 The same procedure was performed using the agar (2 %, w/w) instead of gelatin in order to
223 mimic the exuding wounds. The agar was prepared in the same salt solution as was used for
224 gelatin to emulate wound liquid.

225 The results are presented as the percentage weight gain and corresponding weight loss of the
226 formulation after the test compared with their original weight.

227 All tests were performed in triplicates.

228

2.9. Statistical evaluation

The student's *t*-test was used for comparison of two means. A significance level of $p<0.05$ was considered to be significant.

3. Results and Discussion

3.1 Liposome characteristics

Liposome characteristics are shown in Table 1. The incorporation of rhodamine dyes into liposomes (over 99 % of the starting concentration) resulted in a final dye concentration in liposomes of 0.2 µM for both MP-4 and MTJ-12.

To test whether the charge on the liposome surface is influencing the release of incorporated compounds we prepared liposomes of three different lipid compositions, varying the liposomal surface net charge. PC liposomes exhibited a low positive charge, whereas PC/PG liposomes exhibit a distinguished negative zeta potential and PC/SA liposomes a highly positive charge, respectively (Table 1). The incorporation of dye (Figure 1) into the liposomes resulted in the changes of zeta potential of the negatively charged liposomes (Table 1). It appears that both dyes significantly reduced the negative surface charge of PC/PG liposomes, which can be explained by their positive charge. Dyes become positively charged upon crossing the lipid bilayer due to the change in pH (Figure 2) and are probably electrostatically attracted to the negatively charged surface of PC/PG liposomes, thus reducing their zeta potential.

To determine the effect of liposome size on the release profile of incorporated dyes, vesicles of two distinguished sizes were prepared. The non-sonicated liposomes were clearly of a

multilamellar nature, whereas the sonicated liposomes were probably oligolamellar. The size of sonicated liposomes was found to be similar for the empty liposomes, liposomes containing MP-4 and liposomes containing MTJ-12. Although the size of MP-4 PC/SA seems to be larger compared the other sonicated liposomes, due to their high PI value (0.7), this could be the result of an aggregation of the liposomes rather than the actual sizes of liposomes (Table 1).

3.2. Release of dye from the dye-in-liposomes-in-chitosan hydrogel

The liposomes-in-hydrogel systems represent rather complicated models with respect to the determination of factors which affect the release of liposomally associated active compounds, as well as the choice of method to be used to determine the release. The drug needs to first be released into the hydrogel, followed by its diffusion through the hydrogel and out of the hydrogel. Often, only the amount of drug released from liposomes-in-hydrogel is measured in the acceptor medium (Hurler et al., 2012a). Our goal was to determine the release of drug/dye into the hydrogel and the factors affecting such release. Therefore, our focus was on liposome characteristics, rather than the effects of polymer concentration. It has been previously reported that polymer concentration, especially an increase in polymer concentration, can lead to a decrease in the release of liposome-associated drug as reported for liposomes-in-carbomer hydrogels (Dragicevic-Curic et al., 2009).

The release kinetics of liposome-entrapped hydrophilic compounds from the gels can be determined by liposome characteristics (Mourtas et al., 2008a). In the case of amphiphilic or lipophilic drugs, the lipophilicity of the drug as well as its aqueous solubility will determine the partitioning of the drugs into the aqueous media of the hydrogel (Mourtas et al., 2007). To determine the release of the drug from liposomes incorporated in the hydrogels, the method

originally developed by Peschka et al. (1998) and modified by Pavelic et al. (2001, 2004) and Mourtas et al. (2007) has been reported. However, the method has several limitations. To avoid these limitations, we synthesized pH-sensitive fluorescent dyes to gain a deeper insight into the release of the dye within the gels, avoiding the presence of additional gel as in the agarose method (Peschka et al., 1998).

In this study, the release of two rhodamine dyes, MP-4 and MTJ-12 exhibiting different log P values, namely 4.17 (MP-4) and 2.32 (MTJ-12), respectively, was followed. The role of lipophilicity of drugs on their release from liposomes in liposomes-in-chitosan hydrogels was investigated. We followed the effect of the liposome charge and size and the results are presented in Figure 3.

It is evident that lipid composition influenced the release of dyes out of liposomes and into the chitosan hydrogel. Liposomes with a negative zeta potential (PC/PG) exhibited increased release of both dyes into the hydrogel, whereas liposomes bearing positive charges (PC/SA) had a decreased release of dyes into the hydrogel as compared to the release measured from neutral liposomes (PC) (Figure 3). This was observed for liposomes of both size ranges, namely non-sonicated and sonicated liposomes.

Interestingly, the release of MTJ-12 from PC/PG and PC/SA liposomes reached an early equilibrium state (Figure 3C and 3D); at the beginning MTJ-12 was released from liposomes faster than the more lipophilic MP-4 dye. One possible explanation can be that due to the more hydrophilic character of MTJ-12 (log P = 2.32) compared to MP-4, it diffuses faster into the hydrophilic chitosan hydrogel. However, the neutral liposomes containing MTJ-12 showed a similar release pattern to that seen for the neutral MP-4-containing liposomes. The release of MP-4 during the first 120 min was found to be linearly correlated against the square root of time, which is in agreement with the Higuchi square root law. However, for MTJ-12 the release only followed that law for the first 60 min (data not shown). It appeared that the

301 release of both rhodamine compounds was following a Fickian diffusion in the beginning of
302 the release process, followed by a more steady–state release. Similar observations were
303 reported for lidocaine HCl in the liposomes-in-Carbopol hydrogel systems (Glavas-Dodov et
304 al., 2002) and for griseofulvin in liposomes in both Carbopol- and hydroxyethyl-cellulose-
305 hydrogels (Mourtas et al., 2007).

306 Chitosan hydrogel consists of positively charged chains. Interactions between the positively
307 charged chains and the negatively charged liposomes might disturb the liposomal membrane
308 and facilitate diffusion of the rhodamine dyes out of the liposomes and into the hydrogel.
309 Neutral liposomes are expected to interact less with the chitosan network in the hydrogel, thus
310 the release from those types of liposomes will be slower, as was observed (Figure 3). This
311 could explain why the release from positively charged liposomes was the most sustained of all
312 liposomes tested. Namely, positively charged liposomes are repelled by the chitosan chains,
313 leaving the liposomal membrane undisturbed and preventing the dye molecules from diffusing
314 into the hydrogel. The hydrogel matrix is also expected to protect liposomes from the
315 influence of other excipients within the hydrogel (Mourtas et al., 2008b).

316 Mourtas et al. (2007) proposed that liposomes act as reservoirs that hold lipophilic drugs in
317 gels and release them at the rate determined by the total amount of drug present in the gel. It
318 was also suggested that the diffusion of the released drug through the hydrogel is not the rate-
319 limiting factor as it is faster than the release from liposomes into the hydrogel. DiTizio et al.
320 (2000) found that liposomes composed of dipalmitoylphosphatidylcholine (DPPC),
321 distearoylphosphatidylglycerol (DSPG) and cholesterol in various ratios had varying degrees
322 of affinity for the gelatin-based gel matrix. Similar findings were reported by Liu et al.
323 (2012), who stated that the integrity of liposomes and the subsequent release profile of
324 entrapped calcein is determined by hydrophobic interactions between poly(N-
325 isopropylacrylamide) hydrogel and liposomes. Cohen et al. (2012) showed a correlation

between the lipid composition of liposomes, their leakage stability and subsequently their release properties.

Liposome size did not influence drug release from liposomes bearing neutral (PC) and positive (PC/SA) zeta potential as after 5.5 hours the relative fluorescence intensity was about 0.3. However, the release from sonicated liposomes reached equilibrium faster than non-sonicated ones (Figure 3B). Smaller liposomes with negative zeta potential (PC/PG) exhibited sustained release compared to the bigger, non-sonicated liposomes of the same charge (Figure 3).

This is in agreement with Ruel-Gariepy et al. (2002) who reported a slower release of liposomally-entrapped hydrophilic carboxyfluorescein from chitosan-β-glycerophosphate hydrogel when liposome size was increased from 100 to 280 nm. Neutral liposomes are not expected to get involved in electrostatic interactions with chitosan molecules. However, hydrophobic interactions may take place (Ruel-Gariepy et al., 2002).

It is expected that the large liposomes will release the incorporated drug into the hydrogel in a manner controlled either by the degradation of the chitosan matrix or by the long term destabilization/degradation of the lipid bilayers within the liposomes, depending on the drug's lipophilicity (Ruel-Gariepy et al., 2002). The difference in the lipophilicity did affect the release properties of the rhodamine dyes to the certain extent. After about 75 min, MTJ-12 PC/PG ns preparations reached equilibrium and the fluorescence did not increase after that time point (Figure 3C). In contrast, MP-4 preparations did not reach equilibrium until after more than 120 min (Figure 3A, B). MP-4 is more lipophilic than MTJ-12 and seems to diffuse slower through the lipid bilayer of the liposomes into the hydrophilic gel as compared to the more hydrophilic compound MTJ-12.

Even though the determination of drug release from liposomal hydrogels is rather complex, it is possible to manipulate/optimize the release of drugs from the liposomes into the hydrogel, and subsequently from the hydrogel. The physicochemical interplay between lipophilicity of the drug, liposomal lipid composition and liposomal size, in combination with the properties of the hydrogel has to be taken into consideration.

3.3 Characterization of hydrogels

Hydrogels that are used in therapy are often mixtures of several ingredients such as drug, drug vehicle (polymer) and humectants. These additives may change the textural and rheological properties of the hydrogel, affecting their performance *in vivo* (Hurler et al., 2012b).

Influence of liposomes on rheological properties and texture

In this study we have focused on the rheological characterization of liposomes-in-hydrogels, and the results are shown in Figure 4 (A and B). The plain Carbopol hydrogel exhibited higher shear stress levels at increasing shear rate compared to the liposomal Carbopol hydrogel. A similar observation was reported by Pavelic et al. (2001). Carbopol gels behave predominantly as the elastic solids and have unique rheological properties compared to the other types of gels (Mourtas et al., 2007). It is known that an increase in carbomer concentration induces the domination of elastic over viscous behavior in hydrogels (Dragicevic-Curic et al., 2009). The concentration used in our experiments (0.5 %, w/w) was optimal with respect to the planned application of the hydrogels, i.e. on wounds. The pH is known to affect the hydrogel swelling as well as the rheological and texture properties of Carbopol hydrogels; however the pH in our experiments was maintained in the neutral range as reported earlier (Hurler et al., 2012b). Moreover, we incorporated the non-charged liposomes into the Carbopl hydrogels. Therefore, the resulting changes in the rheological and

texture properties upon the addition of liposomes could thus be attributed to the liposomes rather than to a change in the pH. PC liposomes are in the fluid state and easily deformed under stress conditions, resulting in lower modulation of the rheological properties of the blank gel. Saturated PC on the other hand is in the gel state at the temperature of the measurements and more rigid, thereby changing the rheology of the gel to a greater extent (Mourtas et al., 2008b). Mourtas and coworkers (2008b) showed that PC liposomes changed the viscosity of Carbopol 974 NF hydrogel to a lesser extent than did the hydrogenated PC liposomes, and concluded that liposome composition, not size, affected the rheology of liposomal hydrogels. The incorporation of positively-charged and sterically stabilized liposomes at 2 mM lipid concentration had no effect on the rheological properties of Carbopol gels, whereas gel viscosity was significantly increased in the presence of positively-charged liposomes at 10 mM lipid concentration (Boulmedarat et al., 2003).

The textures of Carbopol hydrogels were shown to be very robust in spite of the addition of increased quantities of PC liposomal dispersion (Hurler et al., 2012b). In our previous study we showed that textures did not change significantly upon the addition of up to 15% (w/w) of PC liposome dispersion (Hurler et al., 2012b). However, the effect of charged liposomes might be pronounced and remains to be evaluated. The chitosan hydrogel texture was shown to be more affected by the inclusion of various additives in the original composition of the gel. However, the addition of glycerol improved the texture properties of chitosan hydrogel, while the addition of 10% (w/w) liposome dispersion led to even further improvement in the texture properties of chitosan hydrogels (Hurler et al., 2012b). However, the incorporation of liposome dispersions into chitosan hydrogel not containing glycerol resulted in the hydrogel exhibiting lower cohesiveness as compared to plain chitosan hydrogel (Hurler et al., 2012b). Glycerol is known to be able to alter the qualities of hydrogen bonds within hydrogels and can thus influence the hydrogel's properties (Islam et al., 2004). Ruel-Gariepy et al. (2002) also

reported that the addition of liposomes into chitosan-β-glycerophosphate gel increased gel strength up to a liposomal concentration of 15 μmol/ml; any further increase in the liposome concentration resulted in a decrease of the original gel strength.

Influence of liposome composition on the texture of chitosan hydrogel

In the experiments discussed above we incorporated liposomes made of plain phosphatidylcholine, as the aim was to develop the simplest formulations possible. However, very often the lipid compositions of liposomes will contain other lipids; some of them charged, resulting in a different surface charge and zeta potential for the liposomes. We attempted to investigate to which extent the zeta potential of liposomes influences the texture of liposomes-in-chitosan hydrogels. Furthermore, we also evaluated whether the size of liposomes affects the texture of liposomes-in-chitosan hydrogels. The results were expressed as the change in gel hardness, cohesiveness and adhesiveness and are shown in Table 2. As can be seen, liposomes stabilized hydrogel hardness, cohesiveness and adhesiveness. Interestingly, smaller liposomes increased these effects significantly more compared to bigger, non-sonicated liposomes, thus appearing to be advantageous with regard to the texture of the resulting liposomes-in-chitosan hydrogels. The surface charge of the incorporated liposomes was found to be affecting the textures of the hydrogels. Compared to the neutral PC liposomes, both PC/PG and PC/SA liposomes increased the hardness, cohesiveness and adhesiveness of the liposomes-in-hydrogel systems significantly. The charged liposomes seem to be able to stabilize the positively charged hydrogel network of chitosan in a better way than the non-charged liposomes (Table 2).

3.4 The absorption- and desorption abilities of hydrogels

Hydrogels destined for the treatment of wounds, especially burns, encounter not only the challenges regarding the efficacy of the active pharmaceutical ingredient and its sustained/controlled release over the desired period of time, but also from the characteristics of the wounds themselves, such as either the strong exuding wounds or dry wounds. It is well recognized that a moist wound environment provides the best healing opportunities. However, at the same time too much moisture can lead to maceration and poor wound healing. The dressing is expected to provide an optimal moist environment and, ideally, be applicable to various types of wounds (Fulton et al., 2012).

Different methods for the evaluation of the exudate handling properties of various wound dressings have been developed. Some methods simply imply the submersion of the dressing into an artificial wound exudate and measure the absorption of fluid into the dressing (Fulton et al., 2012). This method is not suitable for the evaluation of hydrogels. Other methods use more complicated wound models that are combined with a pump system in such a way that the system can be modified regarding the test-fluid and flow rate of the artificial wound exudate (Thomas and Fram, 2001).

A more standardized approach to test the fluid handling ability of the hydrogel dressings is the European norm, "BS EN 13726-1:2002 Test methods for primary wound dressings. Part 1 Aspects of absorbency, Section 3.4, Fluid affinity of amorphous hydrogel wound dressings." Under these controlled conditions hydrogels are exposed to the models of both dry and moist wounds, followed by the determination of the fluid uptake or fluid release/donation, respectively. We have applied this method to evaluate the hydrogels and the results are presented in Figure 5.

Plain Carbopol hydrogel exhibited a very low fluid uptake, absorbing only approx. 1% of its own weight. The liposomes-in-Carbopol hydrogel did not take up any fluid at all (Figure 5). Similarly, when exposed to gelatin, which was used to mimic the dry wounds, Carbopol-based hydrogels did not show to contribute the fluid. Moreover, Carbopol hydrogel became liquefied and is evidently not applicable for administration onto the wounded site. This finding is supported by earlier reports by Pavelic et al. (2001), who stated that Carbopol hydrogel was losing its original viscosity when coming into contact with buffers. Anionic Carbopol resin is not compatible with the cationic ingredients (sodium ions) of the buffers. The same phenomenon seemed to apply to the present study as the wound models contained Solution A, which mimics physiological conditions.

In contrast, the chitosan-based hydrogels, both plain and liposomal, exhibited high fluid uptake from agar gel, which was used as a model for an exuding wound (Figure 5). The liposomes-in-chitosan hydrogel took up more than 60 % of the artificial wound fluid with respect to its original weight, whereas the plain chitosan hydrogel took up even more (65 %). Chitosan hydrogels were also able to donate fluid to compensate the lack of moisture in the dry wounds. The plain chitosan hydrogel lost about 5% of its original weight while the liposomes-in-chitosan hydrogel contributed 10 % of its original weight to the mimicked dry wound (Figure 4). The liposomes-in-chitosan hydrogel formulations were therefore shown to have the ability to handle both exuding and dry wounds accordingly.

4. Conclusions

Two newly synthesized pH-sensitive rhodamine derivatives of different lipophilicities, fluorescent when in the free form released from liposomes into the hydrogel, but not when

incorporated within liposomes, were shown to provide deeper insight on the drug release from liposomes-in-hydrogel destined to be applied as wound dressings. The release was found to be affected by the lipophilicity of the dyes and liposomal surface charge. The liposomes-in-hydrogel system provided both superior texture properties and the potential to achieve a controlled release of the dye. The texture properties were affected by the liposome charge. The evaluation of the ability of the system to handle wound exudates revealed that the chitosan-based hydrogels were superior to Carbopol-based hydrogels. The liposomes-in-chitosan hydrogel delivery system has therefore the potential to be used as a modern wound dressing.

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References:

BS EN 13726-1:2002 Test methods for primary wound dressings. Part 1 Aspects of absorbency, Section 3.4, Fluid affinity of amorphous hydrogel wound dressings.

Alsarra, I.A., 2009. Chitosan topical gel formulation in the management of burn wounds. Int. J. Biol. Macromol. 45, 16-21.

492 Aoyagi, S., Onishi, H., Machida, Y., 2007. Novel chitosan wound dressing loaded with
493 minocycline for the treatment of severe burn wounds. Int. J. Pharm. 330, 138-145.

494 Bhattarai, N., Gunn, J., Zhang, M., 2010. Chitosan-based hydrogels for controlled, localized
495 drug delivery. Advanced drug delivery reviews 62, 83-99.

496 Boateng, J.S., Matthews, K.H., Stevens, H.N., Eccleston, G.M., 2008. Wound healing
497 dressings and drug delivery systems: a review. J. Pharm. Sci. 97, 2892-2923.

498 Boulmedarat, L., Grossiord, J.L., Fattal, E., Bochot, A., 2003. Influence of methyl-beta-
499 cyclodextrin and liposomes on rheological properties of Carbopol 974P NF gels. Int. J.
500 Pharm. 254, 59-64.

501 Cohen, R., Kanaan, H., Grant, G.J., Barenholz, Y., 2012. Prolonged analgesia from Bupisome
502 and Bupigel formulations: from design and fabrication to improved stability. J. Control.
503 Release 160, 346-352.

504 Denis, T.G.S., Dai, T., Huang, Y.-Y., Hamblin, M.R., 2012. Wound-Healing Properties of
505 Chitosan and Its Use in Wound Dressing Biopharmaceuticals, in: Sarmento, B., das Neves, J.
506 (Eds.), Chitosan-Based Systems for Biopharmaceuticals: Delivery, Targeting and Polymer
507 Therapeutics. John Wiley & Sons, Ltd., pp. 429-447.

508 DiTizio, V., Karlgard, C., Lilge, L., Khoury, A.E., Mittelman, M.W., DiCosmo, F., 2000.
509 Localized drug delivery using crosslinked gelatin gels containing liposomes: factors
510 influencing liposome stability and drug release. J. Biomed. Mater. Res. 51, 96-106.

511 Dragicevic-Curic, N., Winter, S., Stupar, M., Milic, J., Krajisnik, D., Gitter, B., Fahr, A.,
512 2009. Temoporfin-loaded liposomal gels: viscoelastic properties and in vitro skin penetration.
513 Int. J. Pharm. 373, 77-84.

514 Fulton, J.A., Blasiole, K.N., Cottingham, T., Tornero, M., Graves, M., Smith, L.G., Mirza, S.,
515 Mostow, E.N., 2012. Wound dressing absorption: a comparative study. Adv. Skin Wound
516 Care 25, 315-320.

517 Glavas-Dodov, M., Goracinova, K., Mladenovska, K., Fredro-Kumbaradzi, E., 2002. Release
518 profile of lidocaine HCl from topical liposomal gel formulation. Int. J. Pharm. 242, 381-384.

519 Hurler, J., Berg, O.A., Skar, M., Conradi, A.H., Johnsen, P.J., Skalko-Basnet, N., 2012a.
520 Improved burns therapy: liposomes-in-hydrogel delivery system for mupirocin. J. Pharm. Sci.
521 101, 3906-3915.

522 Hurler, J., Engesland, A., Poorahmary Kermany, B., Škalko-Basnet, N., 2012b. Improved
523 texture analysis for hydrogel characterization: Gel cohesiveness, adhesiveness, and hardness.
524 J. Appl. Polym. Sci. 125, 180-188.

525 Hurler, J., Škalko-Basnet, N., 2012. Potentials of Chitosan-Based Delivery Systems in Wound
526 Therapy: Bioadhesion Study. J. Funct. Biomater. 3, 37-48.

527 Islam, M.T., Rodriguez-Hornedo, N., Ciotti, S., Ackermann, C., 2004. Rheological
528 characterization of topical carbomer gels neutralized to different pH. Pharm. Res. 21, 1192-
529 1199.

530 Liu, Y., Li, Z.C., Liang, D.H., 2012. Behaviors of liposomes in a thermo-responsive poly(N-
531 isopropylacrylamide) hydrogel. Soft Matter 8, 4517-4523.

532 Mourtas, S., Duraj, S., Fotopoulou, S., Antimisiaris, S.G., 2008a. Integrity of liposomes in
533 presence of various formulation excipients, when dispersed in aqueous media and in
534 hydrogels. Colloids Surf. B Biointerfaces 61, 270-276.

535 Mourtas, S., Fotopoulou, S., Duraj, S., Sfika, V., Tsakiroglou, C., Antimisiaris, S.G., 2007.
536 Liposomal drugs dispersed in hydrogels. Effect of liposome, drug and gel properties on drug
537 release kinetics. Colloids Surf. B Biointerfaces 55, 212-221.

538 Mourtas, S., Haikou, M., Theodoropoulou, M., Tsakiroglou, C., Antimisiaris, S.G., 2008b.
539 The effect of added liposomes on the rheological properties of a hydrogel: a systematic study.
540 J. Colloid Interface Sci. 317, 611-619.

541 Noel, S.P., Courtney, H., Bumgardner, J.D., Haggard, W.O., 2008. Chitosan films: a potential
542 local drug delivery system for antibiotics. Clin. Orthop. Relat. Res. 466, 1377-1382.

543 Paavola, A., Kilpelainen, I., Yliruusi, J., Rosenberg, P., 2000. Controlled release injectable
544 liposomal gel of ibuprofen for epidural analgesia. Int. J. Pharm. 199, 85-93.

545 Pavelic, Z., Skalko-Basnet, N., Filipovic-Grcic, J., Martinac, A., Jalsenjak, I., 2005.
546 Development and in vitro evaluation of a liposomal vaginal delivery system for acyclovir. J.
547 Control. Release 106, 34-43.

548 Pavelic, Z., Skalko-Basnet, N., Schubert, R., 2001. Liposomal gels for vaginal drug delivery.
549 Int. J. Pharm. 219, 139-149.

550 Pavelic, Z., Skalko-Basnet, N., Schubert, R., Jalsenjak, I., 2004. Liposomal gels for vaginal
551 drug delivery, in: Düzgünes, N. (Ed.), Methods Enzymol. Elsevier Academic Press, San
552 Diego, pp. 287-299.

553 Peschka, R., Dennehy, C., Szoka, F.C., Jr., 1998. A simple in vitro model to study the release
554 kinetics of liposome encapsulated material. J. Control. Release 56, 41-51.

555 Ribeiro, M.P., Espiga, A., Silva, D., Baptista, P., Henriques, J., Ferreira, C., Silva, J.C.,
556 Borges, J.P., Pires, E., Chaves, P., Correia, I.J., 2009. Development of a new chitosan
557 hydrogel for wound dressing. Wound Repair Regen. 17, 817-824.

558 Ruel-Gariepy, E., Leclair, G., Hildgen, P., Gupta, A., Leroux, J.C., 2002. Thermosensitive
559 chitosan-based hydrogel containing liposomes for the delivery of hydrophilic molecules. J.
560 Control. Release 82, 373-383.

561 Salam, A., Pawlak, J.J., Venditti, R.A., El-tahlawy, K., 2010. Synthesis and characterization
562 of starch citrate-chitosan foam with superior water and saline absorbance properties.
563 Biomacromolecules 11, 1453-1459.

564 Thomas, S., Fram, P., 2001. The development of a novel technique for predicting the exudate
565 handling properties of modern wound dressings. J. Tissue Viability 11, 145-153, 156-160.

566 Thomas, S., Hughes, G., Fram, P., Hallett, A., 2005. An in-vitro comparison of the physical
567 characteristics of hydrocolloids, hydrogels, foams and alginate/CMC fibrous dressings. S. M.
568 T. L. Report, 1-24.

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570 **List of Figures with legends**

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572 Figure 1. Rhodamine dye derivatives

573

574 Figure 2. Non-charged non-fluorescent spirocyclic form (a) of rhodamine dye incorporated in
575 liposomes at higher pH and positively charged open form (b), formed upon crossing the
576 liposome bilayer, at lower pH.

577

578 Figure 3. Release of MP-4 (A, B) and MTJ-12 (C, D) from phosphatidylcholine liposomes-in-
579 chitosan hydrogel. Both non-sonicated liposomes (filled symbols) and sonicated liposomes
580 (open symbols) were tested. (n=3)

581 * significant vs. PC MP-4 ($p<0.05$); ** significant vs. PC MTJ-12 ($p<0.05$)

582

583 Figure 4. Flow behavior of Carbopol hydrogel with and without incorporated liposomes (A)
584 and chitosan hydrogel with and without incorporated glycerol and liposomes (B). (n=3)

585

586 Figure 5. Absorption and desorption properties of liposomes-in-hydrogels wound dressings.

587 (n=3)

588

589

590 **List of Tables with legends**

591

592 **Table 1. Liposome characteristics (n=3)**

593 * size is an estimate due to PI > 0.7.

594

595 **Table 2. The effect of liposome surface charge and size on the texture of liposomes-in-**
596 **chitosan hydrogel. (n=3)**

597 * significant vs. PC ns ($p<0.05$)

598 ** significant vs. PC s ($p<0.05$)

599 Water (10%, w/w) served as a control. Liposomal dispersions in concentrations of 10 %
600 (w/w) were incorporated into chitosan hydrogel.

601

602

*

List of Abbreviations:

ns = non-sonicated

PC = phosphatidylcholine

PC ns = non-sonicated phosphatidylcholine liposomes

PC s = sonicated phosphatidylcholine liposomes

PC/PG = phosphatidylcholine/phosphatidylglycerol

PC/PG ns = non-sonicated phosphatidylcholine/phosphatidylglycerol liposomes

PC/PG s = sonicated phosphatidylcholine/phosphatidylglycerol liposomes

PC/SA = phosphatidylcholine/ octadecylamine

PC/SA ns = non-sonicated phosphatidylcholine/ octadecylamine

PC/SA s = sonicated phosphatidylcholine/ octadecylamine

PG = phosphatidylglycerol

PI = polydispersity index

s = sonicated

SA = octadecylamine (=stearylamine)