Bioorganic & Medicinal Chemistry Letters 20 (2010) 3021–3025

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Novel Tween® 20 derivatives enable the formation of efficient pH-sensitive drug delivery vehicles for human hepatoblastoma

Andrea Masotti [a,*,†], Paola Vicennati [b,†], Anna Alisi [c], Carlotta Marianecci [d], Federica Rinaldi [d], Maria Carafa [d], Giancarlo Ortaggi [b]

[a] Gene Expression—Microarrays Laboratory, IRCCS-Children's Hospital Bambino Gesù, P.za S. Onofrio 4, 00165 Rome, Italy
[b] Department of Chemistry, SAPIENZA University of Rome, P.le A. Moro 5, 00185 Rome, Italy
[c] Liver Unit, IRCCS-Children's Hospital Bambino Gesù, P.za S. Onofrio 4, 00165 Rome, Italy
[d] Department of Chimica e Tecnologie del Farmaco, Faculty of Pharmacy, SAPIENZA University of Rome, P.le A. Moro 5, 00185 Rome, Italy

ARTICLE INFO

Article history:
Received 1 February 2010
Revised 2 April 2010
Accepted 6 April 2010
Available online 9 April 2010

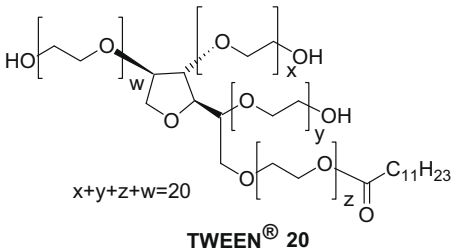
Keywords:
Tween® 20
pH-sensitive vesicles
Drug delivery systems
Hepatoblastoma

ABSTRACT

We describe the synthesis, the physicochemical characterization and the biological evaluation of three novel pH-sensitive systems prepared derivatizing polysorbate 20 (Tween® 20) with glycine, *N*-methyl-glycine and *N*,*N*-dimethyl-glycine (**TW20-GLY**, **TW20-MMG** and **TW20-DMG**). These derivatives form pH-sensitive vesicles and translocate small molecules into cells. The reported systems are efficient drug delivery systems for human hepatoblastoma cells.

Polysorbate 20 (Tween® 20) is a stable non-ionic and non-toxic surfactant widely used in a variety of scientific, pharmaceutical and cosmetic applications.[1] Tween® 20 is a polyoxyethylene derivative of sorbitan monolaurate, distinguished from the other Tween-molecules by the length of the fatty acid ester moiety (Fig. 1).

Non-ionic surfactants are able to form vesicles, called 'niosomes'.[2] Niosomes have a closed bilayer structure analogous to phospholipid vesicles (liposomes). Niosomes share with liposomes the ability to encapsulate hydrophilic as well as lipophilic molecules and therefore they act as drug carriers. In recent years, niosomes made of equimolar amounts of Tween® 20 and cholesterol have been prepared and characterized.[3,4] Despite this, the preparation of charged (pH-sensitive) vesicles is highly desirable for biomedical applications.[5,6] To couple the versatility of niosomes and the properties of pH-sensitive vesicles, two recent papers dealed with the physicochemical and biological characterization of charged niosomes based on Tween® 20 and their interaction with Raw 264.7 (mouse monocyte macrophage).[7,8] These vesicles were prepared using Tween® 20, cholesterol and cholesteryl hemisuccinate (CHEMS). This formulation efficiently interacts with target membranes (i.e., endosomal membrane) and releases the encapsulated material into the cell cytoplasm.[7,8] However, in these systems the pH-sensitivity is achieved with the aid of CHEMS, a pH-reactive molecule employed together with Tween® 20 and cholesterol. Therefore, with the aim of integrating niosomes' delivery properties and pH-sensitivity within the same moiety, we synthesized a novel class of compounds based on Tween® 20 surfactant bearing different pH-sensitive head groups (i.e., glycine, *N*-methyl-glycine, *N*,*N*-dimethyl-glycine). To the best of our knowledge, these pH-sensitive niosomes have never been reported in the literature to date. These novel compounds form pH-sensitive vesicles and we assessed their delivery properties on a human hepatoblastoma (HepG2) cell line. Hepatoblastoma is the most common liver cancer in children.[9] To date, surgical resection represents the best-



TWEEN® 20

Figure 1. Molecular formula of polyoxyethylene-sorbitan monolaurate (Tween® 20).

* Corresponding author. Tel.: +39 06 6859 2650; fax: +39 06 233234321.
E-mail addresses: andrea.masotti@opbg.net, andrea_maso@yahoo.it (A. Masotti).
† These two authors equally contributed to the work.

0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.04.010

choice therapy. In advanced cancer stages with poor prognoses, adjuvant and neoadjuvant chemotherapy have contributed to improve resectability and long-term survival probability.[10] To improve the patient outcome, systems like liposomes or macromolecular drugs have been used for the delivery of small molecules.[11,12] For their peculiar physicochemical characteristics (dimension and permeability), the novel derivatives prepared in this work could represent efficient delivery systems for hepatoblastoma cells. In fact, due to their pH-sensitivity these novel systems may undergo protonation once penetrated into cells. These vesicular structures may destabilize the endosomal membrane as a consequence of the protonation of the numerous amino groups present on the surface ('proton sponge' effect). This effect, even if more evident in polymeric delivery systems,[13] is also active in these novel systems and it may facilitate the release of their inner cargo into the cytoplasm.

To obtain pH-sensitive Tween® 20 derivatives with differently substituted head groups, we decided to functionalize the terminal hydroxyl groups with glycine (**GLY**), *N*-methyl-glycine (**MMG**) and *N*,*N*-dimethyl-glycine (**DMG**).

We synthesized **DMG**[14] following the Eschweiler–Clarke reaction,[15,16] which is commonly used to obtain methyl derivatives of primary or secondary amines (Fig. 2). The crude product was converted into the corresponding hydrochloride form (**DMG·HCl**) and used for the following esterification reaction. The procedure to obtain Tween® 20 derivatives (**TW20-GLY**,[17] **TW20-MMG**,[18] and **TW20-DMG**[19]) is schematized in Figure 3.

Based on potentiometric measurements, the dissociation constant of **TW20-GLY**, **TW20-MMG** and **TW20-DMG** were $pK_a = 8.26$ (±0.04), $pK_a = 8.52$ (±0.02) and $pK_a = 8.39$ (±0.05), respectively. All pK_a values were lower than the corresponding pK_a of glycine ($pK_a = 9.60$),[20] methyl-glycine ($pK_a = 10.05$)[20] and dimethyl-glycine ($pK_a = 9.80$)[20] in the same experimental conditions. This is consistent with the evidence that methyl- or ethyl-esters of glycine (and/or their amino derivatives such as **MMG** and **DMG**) have lower pK_a values with respect to unmodified glycine.[20]

All the derivatives are able to form vesicle suspensions. Unilamellar pH-sensitive vesicles were prepared separately from a fixed amount (15 mM) of **TW20-GLY**, **TW20-MMG** and **TW20-DMG** and cholesterol (Chol) in 1:1, 1:0.75 and 1:0.5 molar ratios by means of the 'film' method.[4,21]

Vesicles made of **TW20-DMG** are generally bigger than those obtained with **TW20-GLY** and **TW20-MMG**. This phenomenon is more clearly observed at pH 7.4, although we noted few exceptions. In fact, the sterically hindered head group of **TW20-DMG** may lead to a more expanded vesicular structure with respect to those formed with **TW20-GLY** or **TW20-MMG**. Moreover, for the **TW20-DMG**/**Chol** system at a cholesterol molar ratio of 0.75, we observed the formation of a vesicle population with the smallest

Table 1
Dynamic light scattering (DLS) and ζ potential measurements of the novel Tween® 20 derivatives at pH 5.5 and pH 7.4

Samples	Mean diameter (nm)		ζ Potential (mV)	
	pH 5.5	pH 7.4	pH 5.5	pH 7.4
TW20-GLY/**Chol** (1:1)	247 ± 7	232 ± 18	−4.5 ± 0.7	−40.3 ± 0.1
TW20-GLY/**Chol** (1:0.75)	247 ± 8	246 ± 13	−5.5 ± 0.2	−39.6 ± 0.7
TW20-GLY/**Chol** (1:0.5)	271 ± 6	262 ± 18	−4.9 ± 0.4	−39.7 ± 0.4
TW20-MMG/**Chol** (1:1)	—	313 ± 16	—	−35.4 ± 0.7
TW20-MMG/**Chol** (1:0.75)	—	263 ± 9	—	−31.9 ± 0.2
TW20-MMG/**Chol** (1:0.5)	—	289 ± 18	—	−34.0 ± 1.4
TW20-DMG/**Chol** (1:1)	500 ± 38	365 ± 19	−7.2 ± 0.3	−49.6 ± 0.2
TW20-DMG/**Chol** (1:0.75)	394 ± 38	176 ± 8	−9.6 ± 1.2	−46.7 ± 0.2
TW20-DMG/**Chol** (1:0.5)	560 ± 35	443 ± 1	−7.1 ± 0.2	−49.2 ± 1.4

Dimensional and ζ potential data for **TW20-MMG**/**Chol** are not reported (—) due to vesicles' structure disruption at pH 5.5.

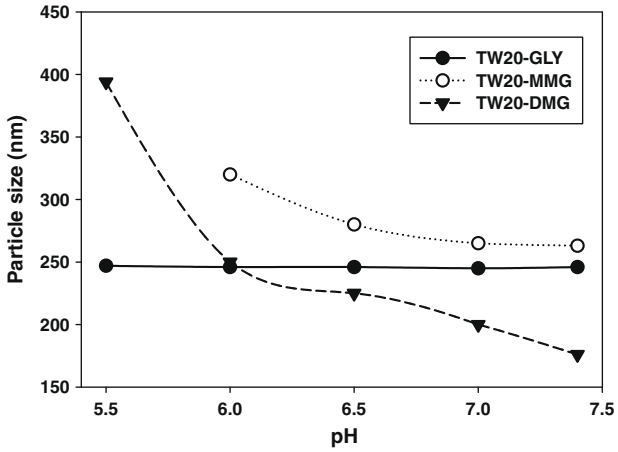


Figure 4. Particle size (nm) variation for **TW20-GLY**/**Chol**, **TW20-MMG**/**Chol** and **TW20-DMG**/**Chol** niosomes at different pH values (5.5 < pH < 7.4). Interpolated lines are a guide for the eyes.

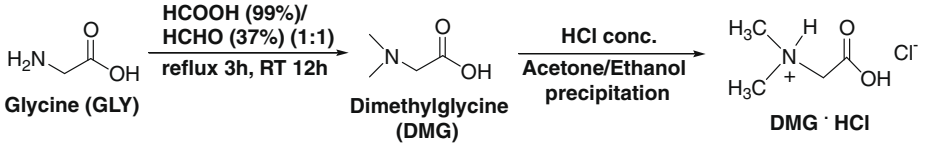


Figure 2. Reaction scheme for the synthesis of dimethyl-glycine (DMG).

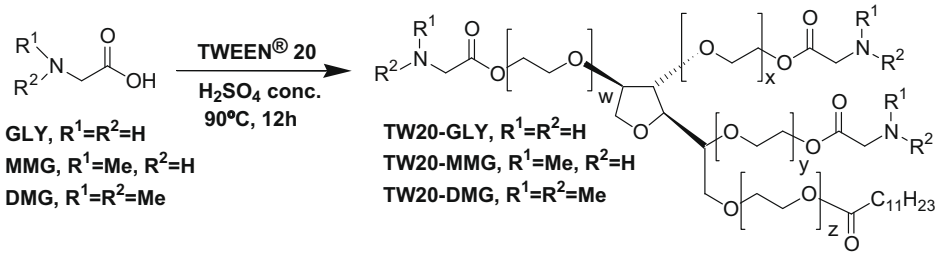


Figure 3. Reaction scheme for the synthesis of pH-sensitive Tween® 20 derivatives **TW20-GLY**, **TW20-MMG** and **TW20-DMG**.

Table 2

Samples	Mean diameter (nm)					ζ Potential (mV)				
	pH 5.5	pH 6.0	pH 6.5	pH 7.0	pH 7.4	pH 5.5	pH 6.0	pH 6.5	pH 7.0	pH 7.4
TW20-GLY/Chol (1:0.75)	247 ± 8	246 ± 7	246 ± 5	245 ± 15	246 ± 13	−5.0 ± 0.2	−25.5 ± 0.3	−31.2 ± 1.4	−34.6 ± 1.5	−39.6 ± 0.7
TW20-MMG/Chol (1:0.75)	—	320 ± 30	280 ± 12	265 ± 9	263 ± 9	—	−10.1 ± 0.4	−13.7 ± 0.4	−19.0 ± 0.9	−34.9 ± 0.7
TW20-DMG/Chol (1:0.75)	394 ± 38	250 ± 26	225 ± 23	200 ± 13	176 ± 8	−9.0 ± 1.2	−25.0 ± 1.3	−23.6 ± 0.5	−32.2 ± 0.6	−46.7 ± 0.2

diameter (176 ± 8 nm) and a low polydispersity index (PDI = 0.29). For **TW20-GLY/Chol** we detected a slight increase in vesicle dimensions as the concentration of cholesterol decreased. It has been previously demonstrated that a decrease in cholesterol content leads to higher bilayer flexibility and, as a consequence, to increased vesicle dimensions.[7,22]

At acidic pH (pH 5.5), the amino (or methylamino) groups are protonated and this generally lead to an increase of niosomes' vesicle size like in the case of **TW20-DMG/Chol**. This may be due to the minimization of electrostatic repulsion on the outer surface and/or to an increase in the vesicle coalescence and fusion phenomenon. The only exception is represented by **TW20-GLY/Chol** vesicles that do not display a significant size increase at this pH value. For **TW20-MMG/Chol** system we were not able to record statistically significant high-quality data at this pH for all the three surfactant/cholesterol ratios (Table 1). This was due to the disruption of vesicular structures at this acidic pH. To study in details this phenomenon, we prepared **TW20-GLY/Chol**, **TW20-MMG/Chol** and **TW20-DMG/Chol** niosomes at a (1:0.75) molar ratio and we determined their size and ζ potential over a gradual incremental change in pH (5.5 < pH < 7.4) (Fig. 4 and Table 2).

At more acidic pH values both **TW20-MMG/Chol** and **TW20-DMG/Chol** systems displayed a gradual increase of particle size. Again, **TW20-GLY/Chol** niosomes displayed no significant variation. In addition, the ζ potential measurements emphasize the pH dependence: at acidic pH (pH 5.5) the ζ potential ranges from −4.5 (±0.7) mV to −9.6 (±1.2) mV while at neutral pH (pH 7.4) it ranges from −31.9 (±0.2) mV to −49.7 (±0.2) mV. As a comparison, vesicles formed by Tween® 20 (a non-pH-sensitive system) displayed an almost constant ζ potential (from −35.7 (±0.8) mV at pH 5.5 to −38.7 (±2.5) mV at pH 7.4).[7] The ζ potential measurements confirm the niosomes' pH-sensitivity and support the hypothesis that the fusogenic tendency observed at pH 5.5 is probably due to the bilayer phase transition from a lamellar phase (pH 7.4) to a hexagonal phase (pH 5.5).[8]

Taken together, all these physicochemical data suggest that these novel pH-sensitive niosomes should display good endosomal escape properties in vivo.

To assess the delivery efficiency of pH-sensitive niosomes, cell interaction studies were performed employing **TW20-GLY/Chol** derivatives on hepatoblastoma cells.[23] For comparison purpose, a solution of sodium calcein and calcein-loaded neutral niosomes made of equimolar (15 mM) amounts of Tween® 20 and Chol

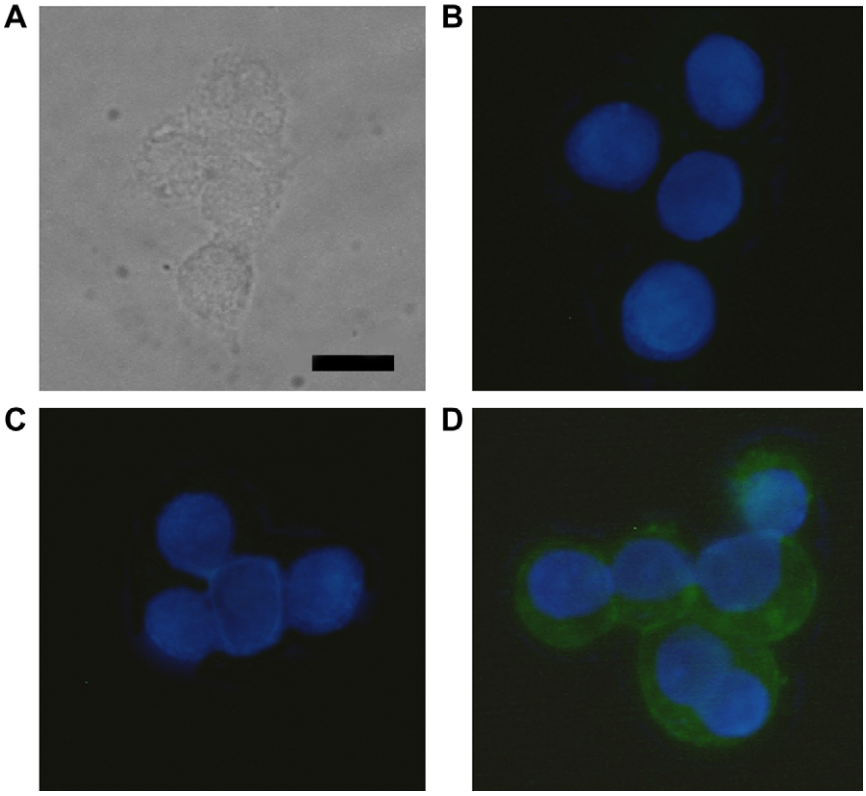


Figure 5. (A) Phase contrast (visible) images of hepatoblastoma (HepG2) cells. (B) Fluorescence image of HepG2 incubated with sodium calcein (10^{-2} M) for 15 min. (C) Fluorescence image of HepG2 incubated with calcein-loaded **TW20/Chol** vesicles for 15 min. (D) Fluorescence image of HepG2 incubated with calcein-loaded **TW20-GLY** vesicles for 15 min. In panels B, C and D nuclei are visualized in blue after DAPI staining. Calcein intracellular delivery (green fluorescence) is visible only in panel D. Scale bar = 10 μm.

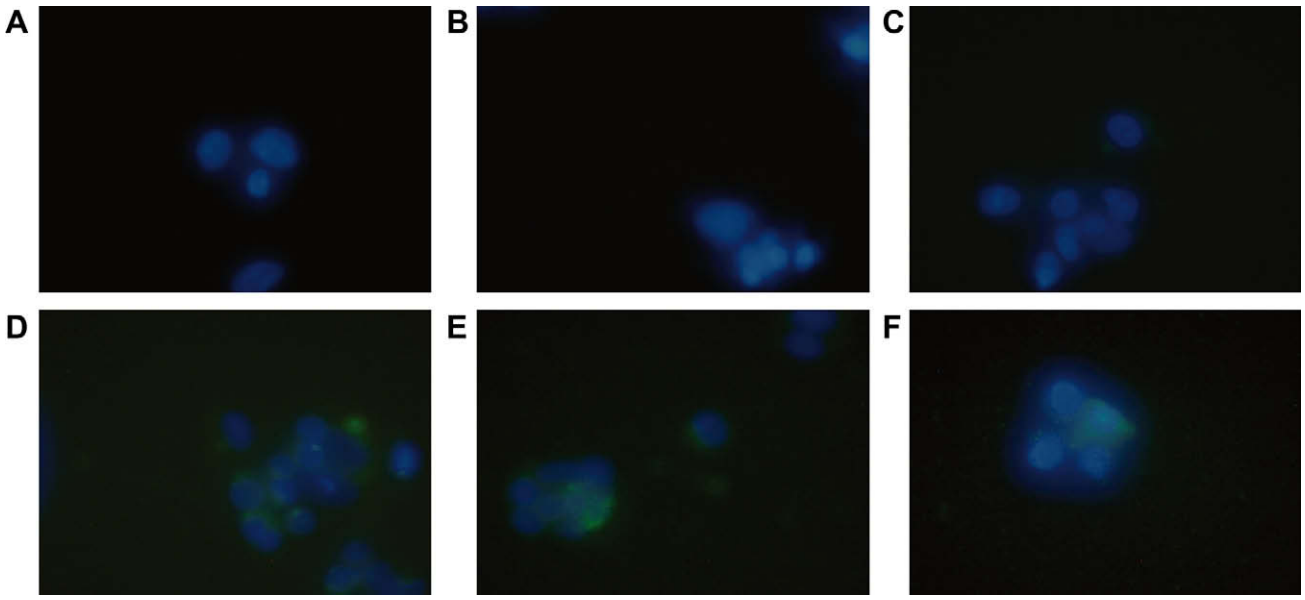


Figure 6. Fluorescence images of HepG2 incubated with calcein-loaded **TW20-GLY**/**Chol** vesicles at (A) 0 min, (B) 5 min, (C) 10 min, (D) 15 min (E) 30 min and (F) 2.5 h. Nuclei are stained with DAPI and visualized in blue while calcein fluorescence is in green. Fluorescence images were merged.

(**TW20**/**Chol**) were also employed. The entrapped calcein concentration resulted 3.5×10^{-5} M for **TW20**/**Chol** and 2.9×10^{-5} M for **TW20-GLY**/**Chol** while the structured surfactant concentration was ~1 mM for both preparations.

Calcein is a carboxylic fluorescent dye unable to enter the cells (Fig. 5B) even after a prolonged incubation (data not shown). Similar results were also obtained for the neutral vesicle formulation **TW20/Chol** in the same conditions (Fig. 5C). The intracellular calcein fluorescence shown in Figure 5D, indicates that **TW20-GLY**/**Chol** vesicles rapidly and efficiently enter the hepatoblastoma cells in 15 min at 37 °C. A time-course fluorescence study indicated that the internalization process occurs between 10 and 15 min (Fig. 6C and D). After 30 min, the fluorescence signal remains constant up to 2.5 h (Fig. 6E and F). All the other derivatives reported in this work displayed good internalization properties and are comparable to the **TW20-GLY**/**Chol** system. The reported niosomes displayed also a low toxicity in the experimental condition used (cell viability >93%).

As expected, all Tween® 20 derivatives synthesized in this work are pH-sensitive. Based on their peculiar acid-base properties, we hypothesize that these systems are able to interact with DNA and other biologically relevant molecules like oligonucleotides, RNA and other charged molecules with therapeutic properties even at physiological pH. The pH dependence of **TW20-GLY**, **TW20-MMG** and **TW20-DMG** is an extremely important property because it has been successfully demonstrated that similar systems are fusogenic and able to enter cells rapidly.[4] The novelty of these derivatives resides in the fact that the vesicle pH-sensitivity has been achieved without the addition of pH-sensitive molecules to Tween® 20 formulations, but with a simple functionalization of the surfactant used for vesicle formation. Herein, we demonstrated the usefulness of the novel pH-sensitive Tween 20 derivatives as delivery vehicles for hepatoblastoma cells. These systems have unique physicochemical and delivery characteristics that make them versatile systems for drug delivery to different tumours.

Acknowledgements

Ministero della Salute and Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR) are gratefully acknowledged for financial funding (PRIN 2005-prot. No. 2005039758).

References and notes

1. Kerwin, B. A. *J. Pharm Sci.* **2008**, *97*, 2924.
2. Uchegbu, I. F.; Vyas, S. P. *Int. J. Pharm.* **1998**, *172*, 33.
3. Santucci, E.; Carafa, M.; Coviello, T.; Murtas, E.; Riccieri, F. M.; Alhaique, F.; Modesti, A.; Modica, A. *STP Pharma Sci.* **1996**, *6*, 29.
4. Carafa, M.; Santucci, E.; Alhaique, F.; Coviello, T.; Murtas, E.; Riccieri, F. M.; Lucania, G.; Torrisi, M. R. *Int. J. Pharm.* **1998**, *160*, 51.
5. Momekova, D.; Rangelov, S.; Lambov, N. *Methods Mol. Biol.* **2010**, *605*, 527.
6. Karanth, H.; Murthy, R. S. *J. Pharm. Pharmacol.* **2007**, *59*, 469.
7. Carafa, M.; Di Marzio, L.; Marianecci, C.; Cinque, B.; Lucania, G.; Kajiwara, K.; Cifone, M. G.; Santucci, E. *Eur. J. Pharm. Sci.* **2006**, *28*, 385.
8. Di Marzio, L.; Marianecci, C.; Cinque, B.; Nazzarri, M.; Cimini, A. C.; Cristiano, L.; Cifone, M. G.; Alhaique, F.; Carafa, M. *BBA Biomembranes* **2008**, *1778*, 2749.
9. Litten, J. B.; Tomlinson, G. E. *Oncologist* **2008**, *13*, 812.
10. Finegold, M. J.; Egler, R. A.; Goss, J. A.; Guillerman, R. P.; Karpen, S. J.; Krishnamurthy, R.; O'Mahony, C. A. *Liver Transpl.* **2008**, *14*, 1545.
11. Gabizon, A. A.; Shmeeda, H.; Zalipsky, S. *J. Liposome Res.* **2006**, *16*, 175.
12. Maeda, H.; Bharate, G. Y.; Daruwalla, J. *Eur J. Pharm. Biopharm.* **2009**, *71*, 409.
13. Masotti, A.; Moretti, F.; Mancini, F.; Russo, G.; Di Lauro, N.; Checchia, P.; Marianecci, C.; Carafa, M.; Santucci, E.; Ortaggi, G. *Bioorg. Med. Chem.* **2007**, *15*, 1504.
14. **DMG**: The reaction consisted in reacting glycine in a (1:1) mixture of formic acid (99%) and formaldehyde (37%) at reflux for 3 h. The one-pot reaction afforded **DMG** in quite good yield (>70%). Mp 182–183 °C; ν(KBr) 1631 cm^{-1}; ^{1}H NMR (300 MHZ, D_2O):δ (ppm) 3.75 (s, 2H), 2.95 (s, 6H); *m*/*c* 104 $(MH)^+$.
15. Eschweiler, W. *Ber.* **1905**, *38*, 880.
16. Clarke, H. T.; Gillespie, H. B.; Weisshauss, S. Z. *J. Am. Chem. Soc.* **1933**, *55*, 4571.
17. **TW20-GLY**: The esterification reaction between Tween® 20 (1 g, mol wt ~1228 Da, ~0.82 mmol) and **GLY** (185 mg, 2.47 mmol) was carried out in the presence of an equimolar amount of H_2SO_4 98% (2.47 mmol, ~200 μl) at 90 °C for 12 h. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 0.84 (t), 1.22 (br), 1.58 (m), 2.06 (s), 2.29 (t), 2.91 (s), 2.99 (s), 3.40 (br), 3.62 (br), 4.18 (m), 7.82 (br). ^{13}C NMR (300 MHz, $CDCl_3$): δ (ppm) 14.00, 22.57, 24.83, 29.05, 29.18, 29.22, 29.37, 29.50, 31.81, 61.57, 63.25, 68.54 (br, several signals), 72.57, 78.83, 80.18, 80.64, 82.56, 84.82, 86.22, 167.49.
18. **TW20-MMG**: The esterification reaction between Tween® 20 (1 g, mol wt ~1228 Da, ~0.82 mmol) and **MMG** (220 mg, 2.47 mmol) was carried out in the presence of an equimolar amount of H_2SO_4 98% (2.47 mmol, ~200 μl) at 90 °C for 12 h. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 0.85 (t), 1.23 (br), 1.56 (m), 2.15 (s), 2.31 (t), 3.42 (br), 3.60 (br), 4.20 (m), 4.82 (br). ^{13}C NMR (300 MHz, $CDCl_3$): δ (ppm) 14.10, 22.77, 24.80, 29.22, 29.24, 29.27, 29.43, 29.46, 31.94, 61.67, 63.38, 68.55 (br, several signals), 72.65, 78.91 (several signals), 86.12, 166.93.
19. **TW20-DMG**: The esterification reaction between Tween® 20 (1 g, mol wt ~1228 Da, ~0.82 mmol) and **DMG·HCl** (346 mg, 2.47 mmol) was carried out in the presence of an equimolar amount of H_2SO_4 98% (2.47 mmol, ~200 μl) at 90 °C for 12 h. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 0.83 (t), 1.22 (br), 1.58 (m), 2.30 (t), 3.06 (br), 3.62 (br), 4.19 (m), 4.86 (br). ^{13}C NMR (300 MHz, $CDCl_3$): δ (ppm) 14.02, 22.59, 24.83, 29.38 (br, several signals), 31.82, 34.13, 44.06, 63.26, 70.46 (br, several signals), 165.44.
20. IUPAC evaluation (25 °C, KCl 0.10 M).
21. Dried films were hydrated with a 10^{-2} M solution of sodium calcein (HEPES, pH 7.4) sonicated (V.C.X 400-Sonics) and purified by size exclusion

chromatography on a Sephadex G75 column. Dynamic light scattering and ζ potential measurements have been carried out using a Malvern Zetasizer Nano ZS90 (Malvern, UK).
22. Uchegbu, I. F.; Florence, A. T. *Adv. Colloid. Interface Sci.* **1995**, *58*, 1.
23. HepG2 cells were seeded onto sterile glass coverslips and placed in 24-well plates. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) at 37 °C under 5% CO_2 and 100% humidity. After reaching 40–50% confluence, cells were incubated with a solution of sodium calcein alone or loaded inside **TW20**/**Chol** and **TW20-GLY**/**Chol** vesicles. All preparations were suspended in complete culture medium. After incubation at 37 °C for 15 min, cells were washed three times with cold phosphate buffered saline (PBS) and fixed by incubation with a methanol/acetone (2:1) solution for 10 min at −20 °C. After three PBS washes, nuclei were stained with 100 μl of a solution of 4′,6-diamidino-2-phenylindole (DAPI) dye (300 nM in PBS) and washed again. Fixed cells were mounted with a glycerol/PBS solution (3:1) to prevent dye photobleaching before fluorescence image acquisition. Images were acquired with a Nikon Eclipse E600 microscope (Nikon, Italy) equipped with epifluorescence optics and a Nikon digital camera DXM1200. Fluorescence images were processed with the dedicated Nikon AC-1 software.