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Mixed micellar system stabilized with saponins for oral delivery of vitamin K

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ABSTRACT

Poorly soluble vitamin K cannot be absorbed by patients suffering from cholestasis due to extremely low level of bile salts in the intestine. A formulation of vitamin K including glycocholic acid (i.e. Konakion® MM), does not increase bioavailability because it is unstable due to protonation of glycocholic acid at gastric pH. To develop a stable formulation, saponins were introduced as neutral surfactants to (partly) replace glycocholic acid. Experimental design was made to investigate the effect of the composition on particle size at neutral pH and upon acidification at pH 1.5. Two formulations that were within the optimized composition window were loaded with vitamin K and those showed superior stability at low pH as compared to Konakion® MM: sizes were between 43 and 46 nm at pH 7.3 and between 46 and 58 nm after 1 h incubation at pH 1.5, respectively, but large aggregates were formed at pH 1.5 in presence of Konakion® MM. Micelles were cytocompatible with Caco-2 cells at concentration of surfactants (saponins and glycocholic acid) up to 0.15 mg/ml. Uptake of vitamin K by Caco-2 cells was 4.2–4.9 nmol/mg protein for saponins-containing formulations and 7.1 nmol/mg protein for Konakion® MM. This, together with the superior stability at low pH, makes saponins-containing mixed micelles promising oral formulations for vitamin K.

1. Introduction

Vitamin K serves as an essential cofactor for γ-glutamyl carboxylase, an enzyme that catalyzes the carboxylation of glutamic acid residues in a number of proteins that are involved in the blood coagulation [1], and plays a key role in haemostasis. The inactivity of the vitamin K-dependent clotting factors VII, IX, X, and prothrombin in case of profound vitamin K deficiency causes spontaneous bleedings, which may be life threatening, particularly in infants with cholestasis due to extremely low level of bile salts and hence poor absorption of vitamin K in the intestine.

Konakion® mixed micelles (MM) is a clinically used formulation composed of vitamin K, egg phosphatidylcholine (EPC or lecithin) and glycocholic acid [2]. It is used for both the prophylaxis and treatment of vitamin K deficiency bleeding (VKDB) in neonates and infants. As reported in earlier studies, prophylactically administering Konakion® MM orally fails to prevent VKDB in cholestatic infants [3], which is thought to be due to the pathophysiological conditions in the upper gastrointestinal tract of those infants. It was found that Konakion® MM are unstable and form large aggregates at the low pH of the stomach because of protonation of the carboxylate group of glycocholic acid, eventually causing coalescence of the formulation [4,5]. Once vitamin K is phase separated in the stomach, its absorption is dependent on the presence of sufficient amount of endogenous bile in the intestine. After passage through stomach in healthy infants, the coalesced vitamin K can be solubilized by endogenous bile in the intestine [6–8]. However, in cholestatic patients, the levels of endogenous bile salts are very low, which in turn results in extremely low absorption of vitamin K from Konakion® MM [9]. Thus, avoiding coalescence and subsequent phase separation of the formulation at gastric low pH is important for sufficient absorption of vitamin K.

To improve the colloidal stability of micellar formulation of vitamin K at gastric low pH, one strategy is to introduce poly(ethylene glycol) [10] as a steric barrier, however these PEGylated mixed micelles displayed a pronounced reduction in both cellular uptake and transport through Caco-2 cell monolayers. PEG is a synthetic polymer and previous studies have demonstrated that PEGylation can indeed reduce the cellular uptake of nanoparticles [11–13]. Another strategy to improve micelles'colloidal stability is to partially replace glycocholic acid in Konakion® MM by a non-ionic surfactant. Saponins, which are of natural sources are chosen as they are biocompatible, safe and cost-effective for scale-up production. Saponins are constituents of a wide variety

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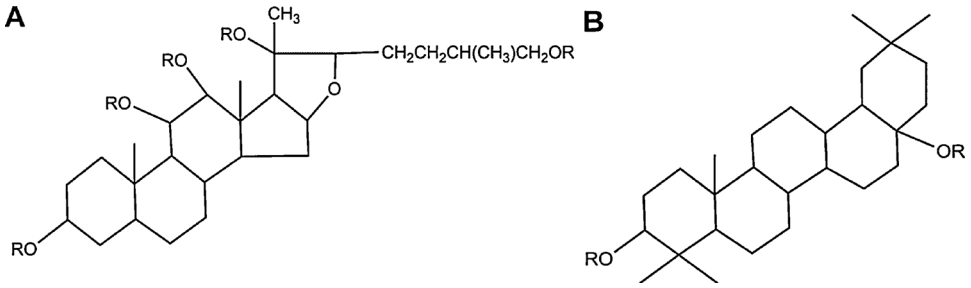

Fig. 1. Chemical structures of (A) steroidal and (B) triterpenoid saponins, R = glucose, galactose, rhamnose, xylose, hexose, etc. [28,29].

of plants (such as quillaja bark and soybean) and consist of polycyclic aglycone as the hydrophobic tail, which is attached to one or several saccharide chains through a glycoside bond. The aglycone part is either a steroid or a triterpene, and the sugar chain can vary in both numbers and length [14–16] (Fig. 1). Saponins can self-assemble into micelles in aqueous solution, in which the non-ionic hydrophilic sugar chains point towards the aqueous phase and the hydrophobic aglycones form the inner core [17–19]. Because saponins used in this study have non-charged sugar moieties (product information from Sigma-Aldrich) [20], and therefore they are pH inert and form micelles with stability independent of the pH [21]. Recently, saponins gained clinical interest because of their membrane permeabilizing properties toward intestinal epithelial cells and these compounds have shown to be able to increase the oral bioavailability of therapeutic drugs [22–25].

The aim of this work was to develop a gastric-stable mixed micellar formulation for oral delivery of vitamin K in which glycocholic acid is partially substituted by saponins aiming to prevent acid induced micellar coalescence. An experimental design approach revealed two mixed micelle formulations that were the most stable at gastric pH and they were selected to load vitamin K. Caco-2 cells were used to investigate the cytocompatibility and cellular uptake of above two mixed micelle formulations of vitamin K containing saponins and glycocholic acid. Cellular uptake by Caco-2 cells is well predictive for epithelial transport [26,27].

2. Materials and methods

2.1. Materials

Lecithin (egg phosphatidylcholine, EPC) was bought from Lipoid GmbH (Ludwigshafen, Germany). Chloroform was purchased from Biosolve (Valkenswaard, the Netherlands). Ethanol was supplied by Merck KGaA (Darmstadt, Germany). Konakion® MM ampoules were purchased from Roche (Basel, Switzerland). Saponins (8–25% sapogenin, product number 84510), sodium glycocholate hydrate, vitamin K, fetal bovine serum (FBS) and all other chemicals and reagents were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Dulbecco's Modified Eagle's Medium (DMEM) was provided by GibCo BRL Life Technologies (Carlsbad, CA, USA). CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit was obtained from Promega (Leiden, the Netherlands). Hoechst dye 33342 was bought from Molecular Probes (Eugene, OR, USA). Syringe filters (0.2 μm) were acquired from Phenomenex (Torrance, CA). Ultrapure water was produced by a Synergy UV water delivery system from Millipore (Billerica, MA, USA).

2.2. Construction of ternary phase diagrams

An extreme vertices design [30] was chosen in order to analyze the effects of mixture components on sizes of mixed micelles at both neutral and acidic pH (7.3 and 1.5, respectively). Ternary diagrams of saponins (S), glycocholic acid (G) and EPC were plotted, each of which represents an apex of the triangle. The weight fractions of S, G and EPC were varied from 0.00 to 0.50 (w/w), 0.00 to 0.50 (w/w) and 0.45 to 0.90 (w/w), respectively (see Section 2.3 for method of preparation of mixed micelle formulations). For all mixtures, the total of S, G and EPC weight fractions always added to 1. Twenty-seven mixtures with varying compositions were prepared (Supplementary material Table S1). The response variables were set as sizes of micelles after preparation at pH 7.3 and after 1 h incubation at pH 1.5, in water at 37 °C, and the corresponding ratio of size $_{(pH\ 1.5)}$ / size $_{(pH\ 7.3)}$. A twenty-seven run design was executed, response variables for each run were recorded (Supplementary material Table S1, all formulations were prepared in triplicate and responses were presented as mean ± SD, n = 3). Ternary diagrams were employed for graphical representations of the obtained response variables using Design-Expert software (Trial Version 10.0, Stat-Ease Inc., MN). The responses were separately fitted into various polynomial models and the statistically valid model was chosen as a best-fit model [30]. The obtained responses were model fitted using analysis of variance in a manner to have (*i*) statistically significant model at 95% confidence interval (*ii*) acceptable R-squared values (> 0.7), (*iii*) difference between adjusted R-squared and predicted R-squared is less than 0.2, and (*iv*) adequate precision value is more than 4 [31,32].

2.3. Preparation of mixed micelle formulations

For the preparation of empty micelles, glycocholate (from 0 to 42 mg) and EPC (from 19–58 mg) were dissolved in 8 ml of chloroform/ethanol (1/1, v/v) in a 100 ml round-bottom flask and the solvents were evaporated under vacuum at 60 °C for 20 min to form a film [33–35]. Next, saponins (from 0 to 42 mg) were dispersed in 5 ml water and this mixture was used to hydrate the film. Next, the dispersions were magnetically stirred for at least 1 h at room temperature, subsequently transferred into a 15 ml Falcon tube (Becton Dickinson) and sonicated for 90 s with 30 s intervals for 6 cycles using a probe sonicator (18% intensity, Cole-Parmer 4710 Ultrasonic Homogenizer, Instrument Co., Chicago, IL). Next, the tubes were immersed in ice cold water to cool down the dispersions during the sonication. Finally, the resulting dispersions were extruded 2 times through a syringe filter (pore size is 0.2 μm). The pH of the above dispersions was adjusted to 7.3 by 1 M NaOH.

Selected stable formulations of mixed micelles (F8 and F9 in Supplementary material Table S1) were loaded with vitamin K. To investigate the vitamin K loading capacity, 38, 50 or 100 μl of vitamin K stock solution (100 mg/ml in chloroform) were added and mixed with EPC (feeding ratio of vitamin K against total lipid was 10.0, 13.2 and 26.3% (w/w), respectively) and glycocholic acid (7.8 or 9.4 mg) to form a film. For the preparation of vitamin K loaded fluorescently labeled mixed micelles, 1.3 ml of 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (rhodamine conjugated PE, Avanti Polar Lipid, Inc) solution in chloroform (117 μM) was added to the mixture. The rest of the procedure was the same as described above for preparation of empty micelles.

Table 1
ANOVA analysis of responses of size$_{(pH\ 1.5)}$, size$_{(pH\ 7.3)}$ and ratio of size$_{(pH\ 1.5)}$/ size$_{(pH\ 7.3)}$ of mixed micellar compositions containing saponins, glycocholic acid and EPC.

Statistical parameters	Responses		
	Size at pH 7.3	Size at pH 1.5	Ratio (size $_{(pH\ 1.5)}$/ size $_{(pH\ 7.3)}$)
Model *p*-value	< 0.0001	< 0.0001	< 0.0001
$R_{squared}$	0.96	0.77	0.91
Adjusted $R_{squared}$	0.95	0.72	0.87
Predicted $R_{squared}$	0.93	0.61	0.75
Adequate precision	31.00	11.65	15.61

2.4. Characterization of mixed micelle formulations

Average size and size distribution of empty micelles at pH 7.3 were determined by Dynamic Light Scattering (DLS, Malvern Instruments, Malvern, UK) at 25 °C and at angle of 90°. The pH of the dispersions (1 ml) was lowered to pH 1.5 with 1 M HCl and the dispersions were subsequently incubated for 1 h at 37 °C. Then particle sizes were determined again by DLS measurements.

Average size and size distribution of vitamin K loaded micelles were determined using DLS. The lipid recovery was determined by measuring the amount of rhodamine conjugated DSPE as described in Section 2.6. Vitamin K loaded micelles (1.0 ml) were diluted with 30 mM HEPES buffer (0.5 ml, pH 7.3) and their zeta potentials were determined by Zetasizer (Malvern Instruments Ltd.). The instrument was calibrated by a standard (DTS1235, −42.0 ± 4.2 mV, Malvern Instruments, UK). The morphology of vitamin K loaded F8K was visualized using Transmission Electron Microscopy (TEM, Tecnai 10, Philips, and 100 kV). The samples for TEM visualization were prepared as follows. A sample of the mixed micelles dispersion (10 µl) was pipetted onto parafilm and a carbon-coated copper grid was placed on the sample for 4 min. Next, the excess liquid was removed by a filter paper and the grid was negatively stained by pipetting a 10 µl droplet of 2% uranyl acetate in demineralized water for 1 min. Next, the excess liquid was removed

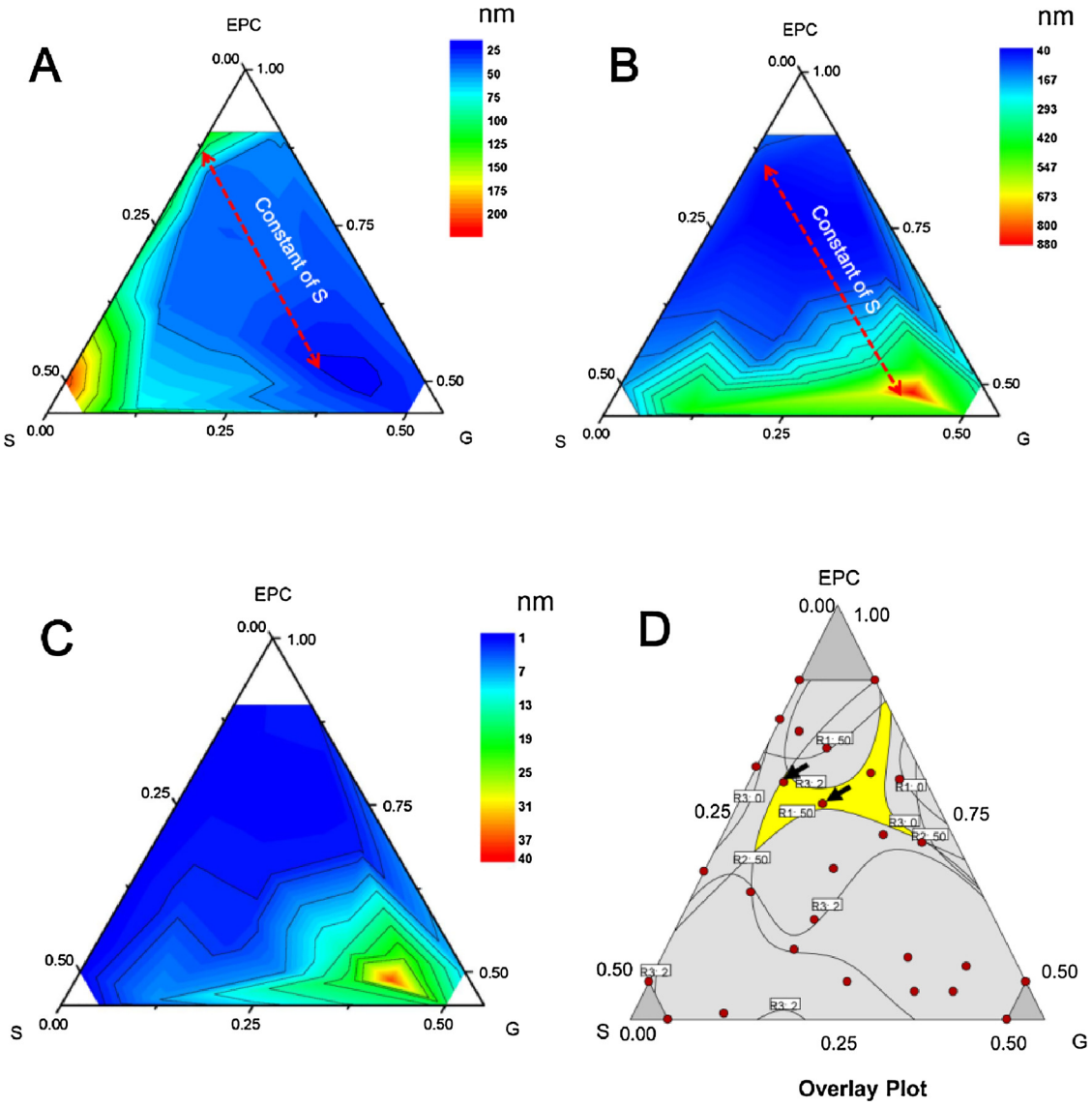

Fig. 2. Formulation optimization of a three-component system of saponins (S), glycocholic acid (G) and EPC; contour plots revealing the effect of independent variables (weight fractions of S, G and EPC) on (A) size at pH 7.3 after preparation (B) size at pH 1.5 after incubating for 1 h at 37 °C and (C) the corresponding ratio (R) of size $_{(pH\ 1.5)}$/size $_{(pH\ 7.3)}$; (D) overlay plot for graphical optimization. Yellow area indicates: sizes of mixed micelles at pH 7.3 and pH 1.5 < 50 nm and the corresponding ratio of size $_{(pH\ 1.5)}$/size $_{(pH\ 7.3)}$ < 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

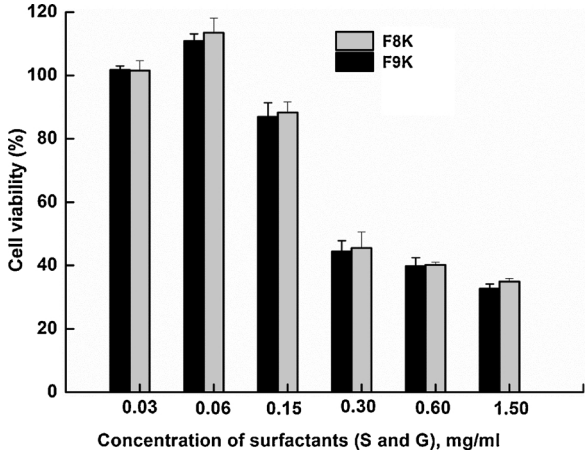

Fig. 3. In vitro cell viability of Caco-2 cells incubated for 36 h with F8K and F9K, one representative experiment in triplicate for each sample, results represent mean ± SD.

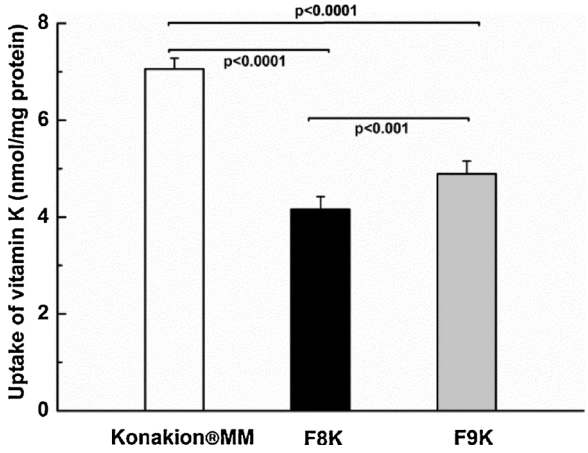

Fig. 4. Uptake of vitamin K upon incubation with Caco-2 cells for 2 h at 37 °C with mixed micelles of Konakion® MM, F8K and F9K at a concentration of 0.24 mM vitamin K, one representative experiment in triplicate for each sample, results represent mean ± SD, $p < 0.0001$ or 0.001 is based on two-tailed unpaired *t*-test.

using a filter paper and the grid was dried for 5 min at room temperature before TEM analysis.

2.5. Stability of vitamin K loaded mixed micelles at low pH

The pH of vitamin K loaded formulations F8 and F9 in water (1 ml) was lowered to pH 1.5 with 1 M HCl and the dispersions were subsequently incubated for 1 h at 37 °C. The size of the micelles was determined by DLS measurements at different time points (0, 10, 20, 40 and 60 min).

2.6. Determination of vitamin K and fluorescent probe concentrations in the formulations

The concentration of vitamin K in different micellar dispersions was determined by RP-HPLC after dilution of the dispersions with ethanol using the same method as described in our previous study [5]. Briefly, a SunFire C_{18} column was used and absorption at 254 nm was used for detection. The mobile phase was ethanol/water (95/5, v/v). The column temperature was 30 °C and the injection volume was 20 µl A calibration curve was obtained using vitamin K dissolved in ethanol with concentrations ranging from 10 to 100 µg/ml.

For determination of the concentration of the fluorescent probe, mixed micelles (100 µl) were dissolved by addition of ethanol (900 µl) and the concentration of rhodamine-conjugated DSPE was determined using fluorescence measurements (FLUOstar Optima, BMG Labtech; for rhodamine conjugated DSPE: $\lambda_{ex} = 560$ nm, $\lambda_{em} = 590$ nm). Calibration curves were obtained using rhodamine conjugated DSPE in ethanol (linear between 1 and 5 µg/ml).

2.7. Cytocompatibility of the mixed micellar formulations

The viability of Caco-2 cells incubated with vitamin K loaded formulations F8K and F9K was evaluated by the MTS assay. In short, Caco-2 cells were seeded in 96-well plates at a density of 1×10^5 cells per well and incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2 for 24 h to allow attachment of the cells to the plates. Next, the medium was removed and 100 µl mixed micelles diluted with blank DMEM (pH 7.4) at different concentrations of total surfactants from 0.03 to 1.50 mg/ml were added to the wells. After 36 h of incubation at 37 °C, the medium was removed and 20 µl Celltiter 96® One Solution was introduced into each well. The plates were subsequently incubated for 2 h at 37 °C, after which the absorbance at 490 nm with a reference wavelength of 655 nm was measured by ELISA microplate reader (Biorad Novapath).

2.8. Cellular uptake of vitamin K

Caco-2 cells (1×10^5 cells per well) were seeded in a 24 well plate and grown in DMEM supplemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2 for 3 weeks to form a confluent monolayer. Subsequently, the cells were incubated with F8K, F9K and Konakion® MM dispersed in blank DMEM at concentrations of 0.24 mM vitamin K for a period of 2 h at 37 °C. Cells were 3 times washed with PBS and subsequently exposed to RIPA lysis buffer (150 mM NaCl, 1.0% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, 2 mM EDTA, pH 8.0) for 10 min at 37 °C and the obtained cell lysates were analyzed with micro BCA protein assay kit (Thermo Fisher Scientific, Perbio Science Nederland B.V) and HPLC to determine the protein and vitamin K concentrations, respectively. Triton X-100 can be used to lyse cells at a concentration between 0.1% and 1.0% [36]. Most frequently used is 1% [37–40], which is higher than its critical micelle concentration values (CMC, reported to be 0.01%-0.02%) [41] to ensure the formation of micelles [42]. This concentration was therefore also used in our study. The HPLC method described by Marinova et al. was used with minor modification [43]. The mobile phase was methanol: ACN: HAc: water (880 ml: 120 ml: 10 ml: 5 ml, in which 1.2 g ZnAc was dissolved). The eluent was flushed with nitrogen gas for 5 min before use. A SunFire C_{18} column was used for separation, and a reduction column (5.5 × 1.5 cm, filled with zinc powder 60–70 µm) was attached to subsequently convert vitamin K into its reduced form to allow fluorescence detection. The flow rate was 1.2 ml/min, the column temperature was 30 °C, and the excitation and emission wavelengths were 246 and 430 nm, respectively. The injection volume was 100 µl A calibration curve was obtained using vitamin K in ethanol (linear between 10 and 500 ng/ml).

For protein quantification, a micro BCA working reagent was prepared by mixing reagents MA, MB and MC (25:24:1, v/v/v) of the Micro BCA Assay Kit. Cell lysates (150 µl) from each well were transferred into the wells of a microplate well. Then, 150 µl of working reagent was added to the wells and mixed using a plate shaker for 30 s. Next, the plate was covered using sealing tape and subsequently incubated at 37 °C for 2 h. Subsequently, the absorbance at 562 nm was measured on a plate reader. A calibration curve was obtained using BSA (bovine serum albumin) solutions with concentrations ranging from 1 to 200 µg/ml.

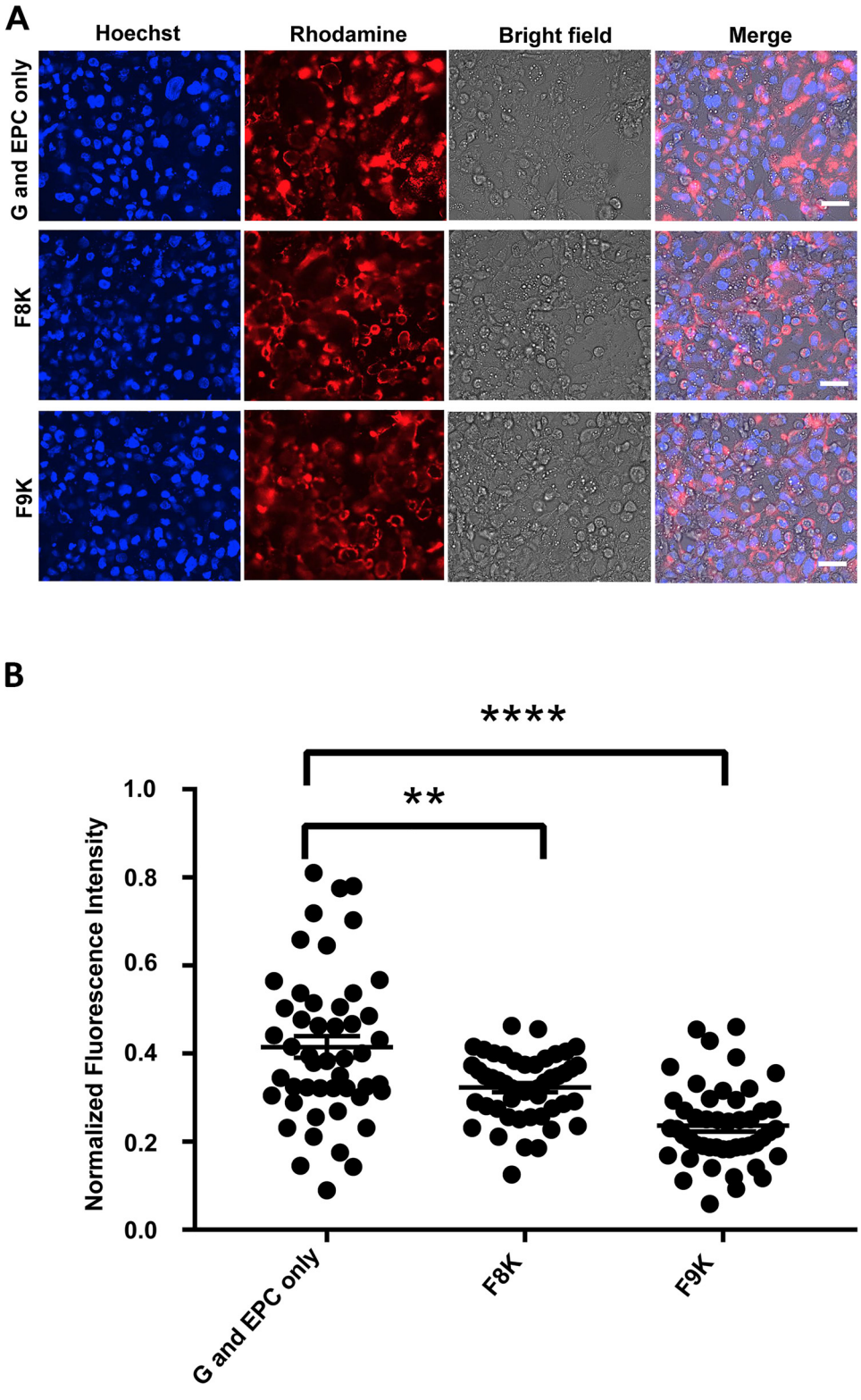

Fig. 5. (A) Confocal laser scanning microscopy pictures of Caco-2 cell monolayers incubated for 2 h with rhodamine labeled vitamin K loaded mixed micelles of G and EPC only, F8K and F9K, all at 37 °C with concentration of 0.24 mM vitamin K. The fluorescence of Hoechst (blue), rhodamine (red), and bright-field image are merged in the right column (scale bar = 50 μm). (B) Normalized fluorescence intensity of rhodamine signal from three different wells for each sample shown in rhodamine channel from confocal laser scanning microscopy pictures, bars indicate average ± SEM, **** $p < 0.0001$ and ** $p < 0.001$ by Kurskal-Wallis test with Dunn's multiple comparisons (right). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

2.9. Cellular uptake of vitamin K loaded rhodamine labeled mixed micelles studied by confocal microscopic analysis

Caco-2 cells (1×10^5 cells per well) were seeded in a 96 well plate and grown in DMEM supplemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2 for 3 weeks to form a confluent monolayer. Next, rhodamine-DSPE labeled vitamin K loaded mixed micelles (100 μl, 5 μM rhodamine-DSPE) were incubated with the cells for 2 h. Subsequently, mixed micelles were removed and the cells were washed with PBS three times. The nuclei of live Caco-2 cells were stained with Hoechst dye 33342 (5 μM in blank DMEM) for 20 min and the monolayers of Caco-2 cells were washed with PBS 3 times and exposed to 150 μl blank DMEM. Next, images were acquired using a high content imaging system (Cell Voyager CV-7000, Yokogawa) with excitation at 405 nm (nuclei staining) and 561 nm (rhodamine staining) at 60 × objective.

2.10. Statistical analysis

Statistical analysis was performed using Prism 7.0 software (GraphPad Software Inc.). Unpaired two-tailed student *t*-test ($p < 0.0001$ or 0.001) or Kurskal-Wallis test with Dunn's multiple comparisons test (**** $p < 0.0001$ and ** $p < 0.001$) were used as indicated in the figure legends.

3. Results and discussion

3.1. Formulation optimization

The aggregation number of bile salts is usually increased when incorporated in mixed micelles, i.e. from 2–10 in form of primary micelles to 10–100 in form of secondary micelles [44,45], which are similar to Konakion® MM that includes a combination of glycocholic acid and EPC. Likewise, Mandal et al. have found that the aggregation number for some small peptides (di-, tri and tetra) and hydantoin drugs can be increased by surfactants [46–52]. In the present work, glycocholic acid was (partly) replaced by saponins to prevent coalescence of the commercial formulation (i.e. Konakion® MM) at gastric pH [4]. The preparation of mixed micelles was based on the method used in our previous publication with minor modification [5]. EPC with or without glycocholic acid was dissolved in chloroform/methanol to form a film after evaporation of the solvent, which was subsequently hydrated by a dispersion of saponins. Saponins are insoluble in chloroform/methanol and therefore they could not be mixed with EPC for formation of the thin film. Without glycocholic acid, micelles with a size above 90 nm were obtained, which were not stable and aggregated upon incubation for 16 h at 20 °C (Supplementary material Fig. S1). The instability of such formulations can be attributed to the existence of out-of-equilibrium and meta-stable structures [53]. Bile salts often help in transformation of such meta-stable formulations into stable micelles [54], and therefore glycocholic acid was selected to obtain small-sized stable micelles. A quality-by-design approach based on extreme vertices experimental design was applied to find the optimal composition giving stable mixed micelles with sizes below 50 nm at pH 7.3 and pH 1.5 and the corresponding ratio of $size_{(pH\ 1.5)}/size_{(pH\ 7.3)} < 2$.

A quadratic model fitted the response values and the model was significant with a p-value < 0.0001 (Table 1), which has relative high predicted $R_{squared}$ and adequate precision (signal to noise ratio). Adequate precision values are greater than 4, implying the suitability of the model. Eqs. (1)–(3) represent the polynomal equations for analysis of size at pH 7.3 after preparation and at pH 1.5 after incubating for 1 h, and the corresponding ratio of $size_{(pH\ 1.5)}/size_{(pH\ 7.3)}$ of various formulations, respectively:

$$Size_{(pH\ 1.5)} = 325\times A + 187\times B + 619\times C - 988\times A\times B - 1927\times A\times C + 828\times B\times C \quad (1)$$

$$Size\ (pH\ 7.3) = 201\times A + 264\times B + 69\times C - 593\times A\times B - 295\times A\times C - 372\times B\times C - 348\times A\times C\times (A\text{-}C) - 324\times B\times C\times (B\text{-}C) - 819\times A\times C\times (A\text{-}C)^2 - 855\times B\times C\times (B\text{-}C)^2 \quad (2)$$

$$Ratio\ [Size\ (pH\ 1.5)\ /\ Size\ (pH\ 7.3)] = -11\times A - 7\times B - 3\times C + 34\times A\times B + 42\times B\times C - 113\times B\times C\times (B\text{-}C) + 94\times A\times B\times (A\text{-}B)^2 + 202\times A\times C\times (A\text{-}C)^2 + 265\times B\times C\times (B\text{-}C)^2 \quad (3)$$

where *A*, *B* and *C* are the weight fractions (w/w) of EPC, saponins and glycocholic acid, respectively. Supplementary material Fig. S2 illustrates that predicted values (also shown in Supplementary material Table S1) according to these equations and the actual measured values were quite close to each other, especially for responses of size at pH 7.3 (Supplementary material Fig. S2 A) and ratio of $size_{(pH\ 1.5)}/size_{(pH\ 7.3)}$ (Supplementary material Fig. S2B). For responses of size at pH 1.5 (Supplementary material Fig. S2C), the predicted values deviated from several measured ones, because micelles composed of F11, F18 and F24-27 were not stable and aggregated upon incubation at pH 1.5 (giving large variation in sizes by DLS shown in Supplementary material Table S1 in bold), which makes the prediction less reliable.

Fig. 2A shows that the size of micelles at pH 7.3 decreased with increasing concentration of G at a constant concentration of S (as shown in dash line in Fig. 2A). Glycocholic acid is inserted into the outermost planar bilayer, and subsequently large lamellar structures are transformed into smaller micelles [5]. For example, size of micelles F11 and F26 are smaller due to relative higher composition of G compared to the sizes of F3 and F4 at pH 7.3 (Supplementary material Table S1). However, Fig. 2B shows that at gastric pH of 1.5, a significant increase in size was observed with an increase of the concentration of G and at a constant concentration of S (as shown in dash line in Fig. 2B). This means that increasing concentration of G makes the micelles unstable at low pH. Correspondingly, the ratio of $size_{(pH\ 1.5)}/size_{(pH\ 7.3)}$ increased from about 1–40 (Fig. 2C), which can be explained by the higher amount of glycocholic acid that results in higher risk of instability of micelles due to protonation of the carboxylate group of glycocholic acid and a subsequent reduction in electrostatic repulsion between the micelles. Fig. 2D shows the overlay plot, generated after selecting the limits of sizes at pH 7.3 and 1.5 below 50 nm, and the corresponding ratio of $size_{(pH\ 1.5)}/size_{(pH\ 7.3)}$ below 2 (yellow area in Fig. 2D). As representative for the optimal formulations for loading of vitamin K, two formulations were selected according to Fig. 2D, with responses that stayed within the optimized area, i.e. F8 and F9 (as indicated by arrows).

3.2. Physicochemical characterization of vitamin K loaded micelles

F8K and F9K are mixed micelle formulations of F8 and F9 loaded with vitamin K. To get insight into the maximum vitamin K loading of the mixed micelles, an increasing amount of vitamin K was added to a fixed amount of total lipid. Large particles with size above 200 nm were obtained when the feeding percentage of vitamin K against total lipid was 13.2% (w/w) and 26.3% (w/w). Supplementary material Table S2 and Supplementary material Fig. S3 present the results of the size distribution and zeta potential of these optimized micelles when the feeding percentage of vitamin K against total lipid was 10.0% (w/w). The average sizes of micelles were 43 and 46 nm (Supplementary material Fig. S3A), and their zeta potentials were -16 to −21 mV (Supplementary material Fig. S3B), respectively. The negative zeta potential of the mixed micelles is caused by the presence of deprotonated carboxylic groups of glycocholic acid at pH 7.3. Vitamin K loaded F9K had a slightly less negative zeta potential (Supplementary material Table S2) because of its relative higher saponins content that caused shielding of the surface charge by the sugar chain of saponins.

With the feeding percentage of vitamin K against total lipid at 10.0% (w/w), the vitamin K recovery, defined as the percentage of recovered vitamin K divided by the added amount of vitamin K, was 85–90% for both micellar formulations F8K and F9K. The corresponding vitamin K loading of the mixed micelles, defined as the percentage mass of vitamin K divided by the total mass of the mixed micelles, was 6.3–7.1%. When rhodamine conjugated DSPE was added to the formulation, its recovery was high as well (89–95%). The vitamin K recovery and vitamin K loading were not measured when the feeding percentage of vitamin K against total lipid was 13.2% (w/w) and 26.3% (w/w) due to instability of the formulations.

The morphology of the particles was investigated by TEM analysis. As formulation of F8K has similar components and size compared to F9K, F8K was selected as a representative formulation for TEM measurements. Fig. S3C shows that small spherical micelles with size below 50 nm were observed, which confirmed the size as determined by DLS in Supplementary material Table S2 and Supplementary material Fig. S3A.

3.3. Stability of mixed micelles at gastric low pH

To investigate whether the introduction of saponins can improve the colloidal stability of glycocholic acid /EPC based vitamin K loaded mixed micelles at gastric pH, the sizes of micelles were studied upon incubation in water at pH 1.5 for 1 h. Mandal and coworkers showed that the critical micelle concentration can be influenced by pH and hence micelle formation is dependent of pH [46–48]. However, the sizes of these vitamin K loaded F8K and F9K changed only from 43.1 ± 1.1 to 45.8 ± 1.4 nm and from 45.9 ± 2.3 to 58.4 ± 2.8 nm, respectively (Supplementary material Fig. S4). The dispersions remained transparent and no coalescence of the formulation was observed, as opposed to Konakion® MM (which size at pH 7.3 is around 7.1 nm, and large aggregates were formed at pH 1.5 (Supplementary material Fig. S5) [4,5]. In the case of saponins based micelles, the sugar moieties of saponins which are at the surface of the micelles (due to their hydrophilic character) likely avoid particle aggregation/coalescence caused by pronation of glycocholic acid at gastric low pH. Therefore, saponins can indeed improve the colloidal stability of glycocholic acid/EPC based formulation at gastric pH for vitamin K loaded micelles.

3.4. Cytocompatibility of saponins containing mixed micelles with vitamin K loading

In order to investigate the cytotoxicity of the saponins containing vitamin K loaded mixed micelles, the viability of Caco-2 cells after incubation with F8K and F9K was determined using the MTS assay. As shown in Fig. 3, the cell viability after 36 h of incubation with F8K and F9K was > 85% with concentration of surfactants (S and G) between 0.03 and 0.15 mg/ml. The cell viability decreased from 85% to 32% when the concentration of total surfactants (S and G) increased from 0.15 to 1.50 mg/ml. The toxic effect of glycocholic acid and saponins at higher levels correlates with previous finding [55,56]. Therefore, F8K and F9K showed good cytocompatibility at concentrations of surfactants (S and G) below 0.15 mg/ml and were further studied for their cellular uptake by Caco-2 cells.

3.5. Uptake of vitamin K loaded rhodamine labeled mixed micelles by Caco-2 cells

As absorption enhancers, both saponins and glycocholic acid can disrupt the lipid arrangements in cell membranes and interact with the polar head groups of the lipid bilayers [24,57]. As a consequence, the lipid membrane becomes fluidized which promotes the diffusion of drugs and even intact micelles across the cell membrane [24,58]. To investigate the uptake of F8K and F9K by intestinal epithelium, Caco-2 cells were selected as a model for small intestinal epithelial cells [59–61]. The commercial formulation Konakion® MM was applied as a positive control. After 2 h incubation with Caco-2 cells at 37 °C, the vitamin K uptake by the cells was 7.1 ± 0.2, 4.2 ± 0.3 and 4.9 ± 0.3 nmol/mg protein for Konakion® MM, and F8K and F9K, respectively (Fig. 4). The uptake of vitamin K was higher for Konakion® MM compared to saponins containing micelles, which is probably because bile salts showed higher permeabilization of Caco-2 cells than saponins [24] and Konakion® MM have a smaller size than saponins containing micelles (about 7 and 43 nm, respectively). However, it is important to note that the uptake of vitamin K was not dramatically reduced when glycocholic acid was partly substituted by saponins.

Furthermore, the cellular uptake of the micelles was monitored by confocal microscopic analysis using mixed micelles that were fluorescently labeled with rhodamine-DSPE to visualize the internalization by and intracellular localization of micelles in Caco-2 cells. The nuclei of Caco-2 cells were stained by Hoechst 33342, which presented blue fluorescence to distinguish from the red fluorescence from the rhodamine-PE labeled micelles (Fig. 5A). The fluorescent signal of rhodamine in red indicates that micelles are taken up by the cells, however the fluorescent intensity of rhodamine from micelles of F8K and F9K (Fig. 5B) displayed relatively lower fluorescence intensity than that from micelles that lack saponins (similar to Konakion® MM), which is consistent with the uptake of vitamin K as shown in Fig. 4.

4. Conclusions

The commercial vitamin K formulation Konakion® MM is unstable and large aggregates are formed at low pH because of protonation of the constituent bile acid, which accounts for the reduced bioavailability under cholestatic conditions. Therefore, improving the stability at low pH was the goal of the present work. In this study, vitamin K loaded mixed micelles composed of saponins, glycocholic acid and EPC were successfully prepared by a film hydration method combined with probe sonication. Saponins-containing formulations, optimized using an extreme vertices design approach, showed good cytocompatibility for Caco-2 cells. Cellular uptake of saponins-containing mixed micelles was slightly reduced as compared to Konakion® MM, however the superior stability at low pH makes it a promising oral formulations for vitamin K for the prophylaxis and treatment of vitamin K deficiency bleeding in neonates and infants suffering from cholestasis.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.colsurfb.2018.06.049.

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