European Journal of Pharmaceutics and Biopharmaceutics 162 (2021) 82–91

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Imine bond formation as a tool for incorporation of amikacin in self-emulsifying drug delivery systems (SEDDS)

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ARTICLE INFO

Keywords:
SEDDS
Imine
Peptide
Amikacin
Hydrophobic aldehydes

ABSTRACT

Aim: The aim was to develop a self-emulsifying drug delivery system (SEDDS) for amikacin via imine bond formation with hydrophobic aldehydes.
Methods: Trans-2, *cis*-6-nonadienal, *trans*-cinnamaldehyde, citral and benzaldehyde were conjugated to amikacin at pH 8.5. Based on results of precipitation efficiency, Fourier-transform infrared spectroscopy (FTIR) and NMR analysis, amikacin-*trans*-cinnamaldehyde conjugates were further characterized regarding log $P_{octanol/water}$ via HPLC. The release of amikacin from the amikacin-*trans*-cinnamaldehyde conjugates was examined through in vitro incubation with bovine serum albumin (BSA). SEDDS containing the amikacin-*trans*-cinnamaldehyde conjugates were tested regarding mean droplet size (MDS), polydispersity index (PDI), log $D_{SEDDS/release\ medium}$ and cell viability.
Results: Trans-cinnamaldehyde formed the most hydrophobic conjugates with amikacin whereas benzaldehyde did not form hydrophobic conjugates at all. Imine bond formation was confirmed by FTIR and NMR analysis. The highest increase in log P was achieved for the amikacin-*trans*-cinnamaldehyde conjugate in a molar ratio of 1:5, shifting from −8.58 up to 1.59. Incubation of this conjugate with BSA led to the formation of BSA-*trans*-cinnamaldehyde releasing in turn amikacin. SEDDS based on Capmul MCM, Cremophor EL and propylene glycol containing the conjugate demonstrated a MDS of 61.4 nm and PDI of 0.265. Log $D_{SEDDS/release\ medium}$ was calculated to be 3.38. Cell viability studies showed very good tolerability of conjugate loaded SEDDS in concentrations of 0.1% − 0.5%.
Conclusion: Imine bond formation of amikacin with *trans*-cinnamaldehyde and the incorporation of the resulting conjugate into SEDDS represents a promising strategy for oral delivery of amikacin.

1. Introduction

Within recent years, self-emulsifying drug delivery systems (SEDDS) have gained a lot of attention as lipid-based vehicles for the oral administration of hydrophilic compounds. By definition, SEDDS are composed of oils, surfactants and co-solvents, forming fine oil-in-water (O/W) emulsions upon dilution in aqueous media through mild agitation [1]. To accomplish the incorporation of hydrophilic compounds into SEDDS, their hydrophobicity has to be increased. This can be achieved by various methods such as hydrophobic ion pairing (HIP), where lipophilic complexes are formed between hydrophilic ionic drugs and lipophilic counter ions based on electrostatic interactions [2,3]. Furthermore, lipophilic complexes can be formed via hydrophobic hydrogen bond pairing (HHP) [4]. Although stable in the lipophilic phase, HIPs and HHPs were shown to be unstable in intestinal fluids [5]. An alternative strategy to generate more stable lipophilic forms of hydrophilic drugs is the covalent attachment of lipophilic ligands via esterification, amidation, REAL lipidization or cyclization [6]. As these chemical modifications can have a significant impact on the therapeutic efficacy of these drugs, however, their applicability is rather limited [4]. In contrast, imine bonds might on the one hand be sufficiently stable in the intestinal fluid and on the other hand be rapidly cleaved in the

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https://doi.org/10.1016/j.ejpb.2021.03.001
Received 10 September 2020; Received in revised form 27 February 2021; Accepted 2 March 2021
Available online 16 March 2021

systemic circulation or at the target site. So far just one study is witnessing the potential of this strategy. The results of this study clearly demonstrated a cleavable imine bond under simulated serum conditions [7]. In detail, conjugates of polymyxin-cinnamaldehyde showed increased lipophilicity in comparison to the unmodified peptide drug. So far, however, a proof-of-concept has only been provided by one study using a peptide drug.

It was therefore the aim of this study to confirm the potential of this strategy by evaluating its potential for another category of BCS class 3 drugs namely aminoglycoside antibiotics. For this purpose, amikacin as highly hydrophilic drug containing four primary amino groups, 13 hydrogen bond donor groups and 17 hydrogen bond acceptor groups was chosen as model API. Furthermore, four different natural aldehydes namely *trans*-2, *cis*-6-nonadienal, *trans*-cinnamaldehyde, citral and benzaldehyde were utilized for imine bond formation. The resulting amikacin-aldehyde conjugates were characterized in terms of precipitation efficiency along with log P investigations. Since conjugates need to be sufficiently unstable to fall apart at their target site, formed imine conjugates were incubated with a model protein (bovine serum albumin; BSA) at a slightly acidic pH mimicking the pathogenic microenvironment [8]. Finally, the most promising conjugates were incorporated into SEDDS and droplet size, PDI, log D and cell viability were determined.

2. Materials

Amikacin disulfate salt (A1774-Sigma-Aldrich), amikacin European Pharmacopoeia reference standard, *trans*-cinnamaldehyde, *trans*-2,*cis*-6-nonadienal, benzaldehyde, citral, sodium hydrogen carbonate, sodium dihydrogen phosphate, sodium hydroxide, Tween 20, Cremophor EL, bovine serum albumin (BSA), sodium phosphate monobasic dihydrate, 1-fluoro-2,4-dinitrobenzene (for HPLC), trifluoroacetic acid (TFA), Triton X-100, resazurin, sodium chloride, potassium chloride and glucose were purchased from Sigma-Aldrich, Austria. Labrasol, Peceol and Labrafil M1944 CS were obtained from Gattefossé, France. Tween 80 was purchased from Gatt Koller, Austria. Mygliol 840 was purchased from Sasol, Germany. Capmul MCM EP was purchased from Abitec, USA. HEPES was purchased from Carl Roth, Germany. All other chemicals were of analytical grade, pharmaceutical or food grade and were purchased from commercial vendors.

3. Methods

3.1. Precipitation efficiency

Four different aldehydes namely benzaldehyde, citral, *trans*-cinnamaldehyde and *trans*-2, *cis*-6-nonadienal were covalently bound to the available amino groups of amikacin via imine bond formation using a previously published method [7]. The amount of 1 mg of pure amikacin was firstly dissolved in 1 mL of demineralized water using vortex followed by addition of aldehydes in different molar ratios ranging from 1:1 to 1:5. Thereafter, the pH value of all solutions was corrected to pH 8.5 ± 0.5 with 1 M NaOH while shaking on thermomixer (Eppendorf Thermo Mixer® C, Hamburg, Germany) at 400 rpm, 25 °C for 1 h. The resulting conjugates appeared as white precipitate on the bottom of the tube and separation was achieved by centrifugation at 10000 rpm for 20 min (MiniSpin®, Eppendorf, Austria GmbH). Precipitates were washed with water to remove unreacted amikacin. In order to remove unreacted aldehyde, precipitates were washed with diethyl ether. Then the precipitates were frozen at −80 °C for 2 h and lyophilized for 24 h (Christ Gamma 1-16 LSC Freezedryer). The conjugates were stored at −20 °C until further use. Furthermore, the precipitation efficacy was calculated by measuring the concentration of free unreacted amikacin dissolved in the supernatant via TNBS assay (2,4,6-trinitrobenzenesulfonic acid solution) photometrically at 450 nm (Tecan infinite M200 spectrophotometer, Austria) [9]. TNBS is a reagent that reacts with primary amines and forms trinitrophenyl derivates as chromogenic products. Calibration curve of amikacin disulfate salt was used to calculate the precipitation efficiency of the formed imines. A 400 µl aliquot of the methanolic TNBS solution was added to 10 mL of 8% (w/v) $NaHCO_3$ solution. Thereafter, TNBS mixture was added to each supernatant in a ratio of 1:1 (70 µl TNBS mixture + 70 µl supernatant). The mixtures were incubated at 300 rpm and 37 °C for 90 min. Afterwards, the absorbance was immediately measured at 450 nm (Tecan infinite® M200 spectrophotometer, Austria). The precipitation efficiency was calculated by the following equation [9,10]:

$$Precipitation\ efficiency(\%) = 100 - \left(\frac{Amikacin\ concentration\ after\ conjugate\ formation}{Amikacin\ concentration\ before\ conjugate\ formation} * 100\right) \quad (1)$$

To confirm imine bond formation, IR analysis was conducted and compared with the spectrum of educts (amikacin and the different aldehydes). IR spectra were recorded on a Bruker ALPHA FT-IR apparatus.

In order to confirm that all four amino groups of amikacin reacted with *trans*-cinnamaldehyde, ^{1}HNMR spectra of formed conjugate were recorded on a 400 MHz Bruker Avance 4 Neo spectrometer. The centre of the solvent multiplet (DMSO-d_6) was used as internal standard (chemical shifts in δ ppm), which was related to TMS with δ 2.49 ppm.

3.2. Log P determination

In order to evaluate the lipophilic character of formed amikacin-aldehyde conjugates for incorporation into SEDDS, log P was determined using a standard method reported by our research group. Water and octanol were saturated with each other for 24 h. Then, 500 µl of water-saturated octanol was added to 1 mg of amikacin-*trans*-cinnamaldehyde conjugates formed in molar ratios of 1:1–1:5. After vortexing, 500 µl of octanol-saturated water was added and shaken in an Eppendorf thermomixer at 500 rpm and 37 °C for 24 h. The two phases were separated by centrifugation with high speed mini centrifuge at 13400 rpm for 5 min. The amikacin-*trans*-cinnamaldehyde conjugate concentration in both octanol and water phase was quantified via RP-HPLC (Hitachi Elite LaChrom system equipped with L-2130 pump, L-2200 autosampler and L-2400 UV detector). Samples were analysed on a C18 column, using an isocratic elution at 35 °C and a flow rate of 1.000 mL/min over 6 min. A mobile phase composed of 67% A (0.1% v/v TFA in acetonitrile) and 33% B (0.1% v/v TFA in water) was used [7]. For

quantification via HPLC, a calibration curve with increasing amounts of amikacin-*trans*-cinnamaldehyde conjugate (0.002–1.000 mg/mL) was established. Prior to injection, samples of the octanol phase were diluted in 0.1% (v/v) TFA in acetonitrile/isopropanol. Samples of the water phase were injected without any dilution. Conjugates were quantified with DAD (diode array detector) at wavelength of 325 nm and the partition coefficient was calculated using the following equation:

$$LogP = log_{10}\left(\frac{concentration\ of\ amikacin-trans-cinnamaldehyde\ conjugate\ in\ octanol}{concentration\ of\ amikacin-trans-cinnamaldehyde\ conjugate\ in\ water}\right) \quad (2)$$

3.3. Release of free amikacin from amikacin-trans-cinnamaldehyde conjugate

As imine bonds are unstable in acidic media, they are likely destabilized and consequently hydrolysed in pathogenic microenvironments. For the evaluation of release of amikacin from newly formed amikacin-*trans*-cinnamaldehyde, 1 mg of amikacin-*trans*-cinnamaldehyde conjugate was incubated in 1 mL of 4% (m/v) bovine serum albumin (BSA) solution pH 6.8 (Ph. Eur.) while shaking on thermomixer at 500 rpm at 37 °C for 12 h. Aliquots of 50 µl were withdrawn from the solution and replaced with buffer solution. Samples were analysed by using HPLC as described above.

3.4. Selection of solvents for SEDDS preparation

Firstly, 1 mg of amikacin-*trans*-cinnamaldehyde conjugate was randomly dispersed in 20 mg of different organic solvents. For these studies propylene glycol, Mygliol 840, Peceol, Capmul MCM EP, Labrafil M1944 CS, Labrasol, Cremophor EL, Tween 80 and Tween 20 were chosen. The obtained mixtures were homogenized by mixing each of them on the vortex mixer for 5 min. Then, they were sonicated (Bandelin Sonorex at a frequency of 35 kHz) for 2 min and shaken on thermomixer at 25 °C and 1000 rpm for 24 h. Solubility was evaluated via UV/VIS analyses at 600 nm after centrifugation at 13 400 rpm for 5 min.

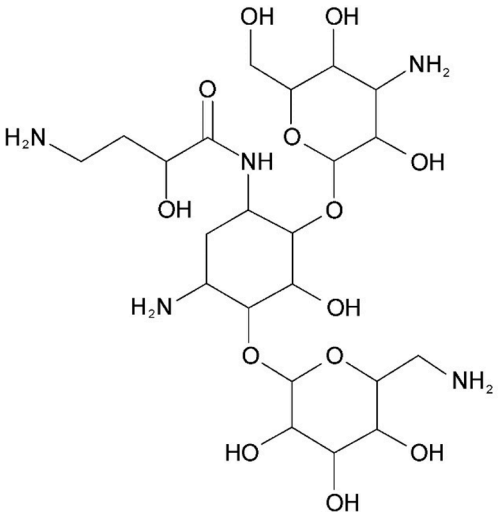


Fig. 1. Chemical structure of amikacin.

3.5. Loading and characterization of SEDDS

Blank SEDDS pre-concentrates were prepared by vortexing lipids, surfactants and co-solvents followed by ultrasonication for 2 min. Loaded SEDDS pre-concentrates were prepared by firstly dissolving the amikacin-*trans*-cinnamaldehyde conjugate in the oily component, followed by the addition of co-solvents and surfactants to the solution. Both blank and loaded SEDDS pre-concentrates were additionally kept on stirring at 25 °C and 1000 rpm for 12 h. Blank and loaded SEDDS nanoemulsions were prepared by diluting SEDDS pre-concentrate at the ratio 1:100 in 20 mM phosphate buffer saline (PBS) pH 6.8 at 37 °C while shaking at 300 rpm. Thereafter, the mean droplet size and the PDI (polydispersity index) of both nanoemulsions were determined by photon correlation spectroscopy with Nano-ZSP (Malvern instruments, Worcestershire, UK) directly after preparation and after 4 h at 37 °C while shaking at 300 rpm. The measurements were conducted at following parameters: viscosity (0.6864 cP), refractive index (1.330), cell (disposable cuvettes –DTS0012), measurement angle (173° backscatter), data processing analysis model (normal resolution), software (Zetasizer Nano ZSP Malvern) and wavelength (4 mV; 637.8 nm).

3.6. Distribution coefficient determination (log D)

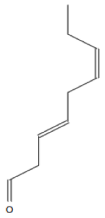
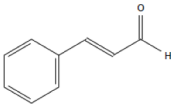
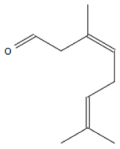
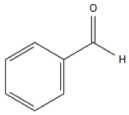
First, 100 mg of chosen SEDDS pre-concentrate formulation was saturated by amikacin-*trans*-cinnamaldehyde conjugate. In parallel, 1 mL of demineralized water was saturated with the conjugate. The dispersions were mixed for 12 h and centrifuged. After centrifugation, the concentration of dissolved amikacin-cinnamaldehyde conjugate in the supernatants of SEDDS and demineralized water was quantified via HPLC. Prior to injection, SEDDS were diluted in 0.1% (v/v) TFA in acetonitrile/isopropanol. Samples dissolved in demineralized water were analysed without any dilution. Log D was calculated according to the following equation [2]:

$$logD\left(\frac{SEDDS}{release\ medium}\right) = log\left(\frac{maximal\ solubility\ in\ SEDDS}{maximal\ solubility\ in\ demineralized\ water}\right) \quad (3)$$

3.7. Cell viability studies

To determine the potential cytotoxic effect of SEDDS, resazurin assay was used to evaluate the cell viability on a human colon carcinoma cell line (Caco-2 cells) [11]. Caco-2-cells were supplied from European collections of cell cultures (ECACC, Health Protection Agency, Proton Down, Salisbury, Wiltshire, UK). The passage of 35–40 was used. Approximately 2.5×10^4 cells per well were seeded to a 24-well plate. Cells were incubated in red MEM supplemented with 10% (v/v) fetal bovine serum (FBS) and penicillin/streptomycin solution (100 units/0.1 mg) at 95% humidity, 37 °C and atmosphere of 5% CO_2 for 14 days. Caco-2 cells were seeded for 14 days in a 24-well-plate in 500 µl of red MEM (minimal essential medium) containing 10% (v/v) fetal bovine serum albumin and penicillin/streptomycin solution (100 units/0.1 mg/L) at 95% humidity, cultivated at 37 °C in 5% CO_2 environment. The medium was replaced every 48 h. After 14 days a cell monolayer was formed and the cell viability study was performed. Caco-2 cells were washed with 500 µl of prewarmed Hanks balanced salt solution (HBSS) and were incubated at 37 °C for 30 min. Chosen SEDDS formulations loaded with 1% amikacin-*trans*-cinnamaldehyde conjugate formed in

Table 1
Aldehydes used for the imine formation with amikacin (Chemicalize.org).

Name	Trans-2,*cis*-6-nonadienal	Trans-cinnamaldehyde	Citral	Benzaldehyde
Chemical structure				
Molecular weight	138.21 g/mol	132.162 g/mol	152.237 g/mol	106.124 g/mol
Formula	$C_9H_{14}O$	C_9H_8O	$C_{10}H_{16}O$	C_7H_6O
Aromatic or aliphatic	Aliphatic	Aromatic	Aliphatic	Aromatic
Intrinsic solubility	0.154 mg/ml	1.73 mg/ml	0.338 mg/ml	5.80 mg/ml
Log P	2.62	1.98	2.66	1.69

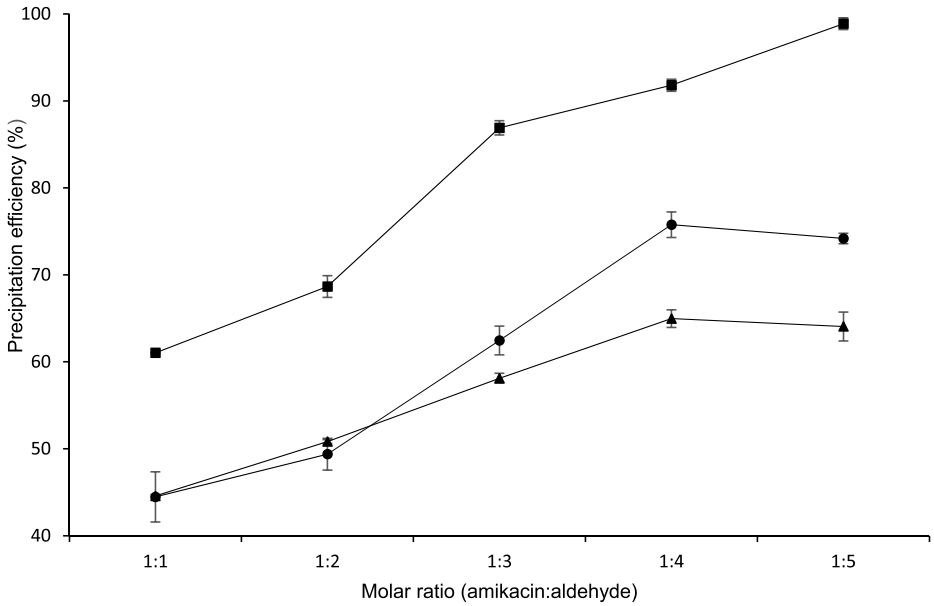


Fig. 2. Precipitation of amikacin (1 mg/ml) by reaction with *trans*-cinnamaldehyde (■), *trans*-2, *cis*-6-nonadienal (●) and citral (▲) in molar ratio 1:1, 1:2, 1:3, 1:4 and 1:5 quantified via TNBS test photometrically. Data are shown as mean ± SD (n = 3).

molar ratio 1:5 were dispersed at concentrations of 0.1%, 0.3% and 0.5% in HBSS and tested on Caco-2 cells. The experiments were performed in triplicate. Caco-2 cells with only HBSS were used as negative control, while cells with 0.1% Triton X-100 in HBSS were used as positive control. All samples were incubated at 37 °C for 4 h. After incubation the SEDDS dispersions and Triton X-100 in HBSS solution were removed to wash the cells with HBSS. Afterwards 200 µl of resazurin solution (20 times diluted in HBSS) was added to each vial. Cells were incubated for additional 3 h. Then, aliquots were transferred to a microplate reader (Tecan infinite® M200 spectrophotometer, Austria) and the fluorescent intensity was measured at an excitation wavelength of 540 nm and an emission wavelength of 590 nm. Cell viability was calculated as follows [12]:

$$Cell\ viability(\%) = \frac{As}{Ac} * 100 \quad (4)$$

where As = fluorescent intensity of SEDDS dispersion and Ac = fluorescent intensity of negative control.

3.8. Statistical analysis

Statistical data analysis was performed by using SPSS software. One-way ANOVA followed by multiple comparison Tukey test was performed. Independent samples *t*-test was used when equality of means between the two groups was analysed. One sample test was used when the mean of a single group was compared to known mean. All statistical analyses were conducted with $p < 0.05$ level of significance. All experiments were performed in triplicate and the values are expressed as means ± SD.

4. Results and discussion

4.1. Precipitation efficiency

Amikacin is an aminoglycoside, having just negligible or not at all resistance to gram-negative, aerobic and gram-positive bacteria [13], typically prescribed to patients who suffer from cystic fibrosis (CF) as a treatment and prevention of chronic infections caused by pathogens such as *Pseudomonas aeruginosa* [14]. As it belongs to BCS class 3 drugs, low membrane permeability causes insufficient oral bioavailability. The development of an oral formulation for amikacin is therefore of interest [15,16]. As amikacin bears four primary amino groups as displayed in Fig. 1, a discrepancy in precipitation efficiency with various molar ratios of amikacin towards used aldehydes was expected. This assumption was confirmed by our experiments. In order to be incorporated in SEDDS, its lipophilicity was increased by conjugation with lipophilic aldehydes as

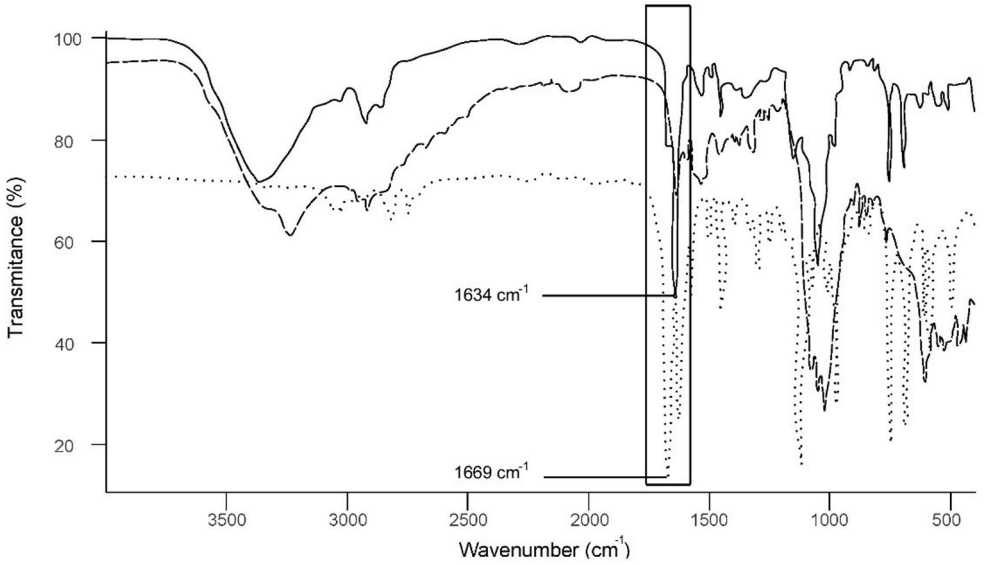


Fig. 3a. Infrared spectrum of amikacin (broken line), *trans*-cinnamaldehyde (dotted line) and amikacin-*trans*-cinnamaldehyde conjugate formed in molar ratio 1:4 (bold line).

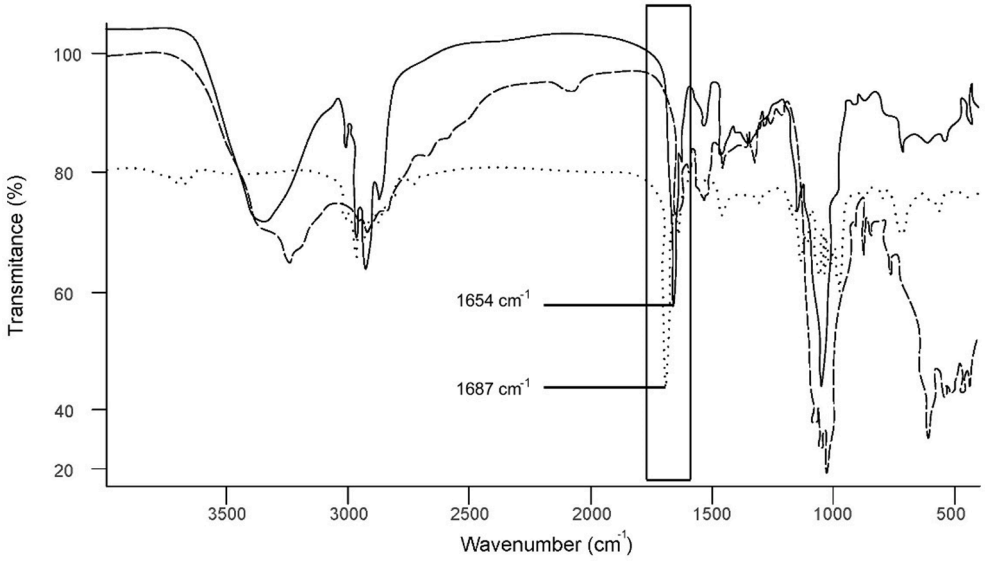


Fig. 3b. Infrared spectrum of amikacin (broken line), *trans*-2, *cis*-6-nonadienal (dotted line) and amikacin-*trans*-2,*cis*-6-nonadienal conjugate formed in molar ratio 1:4 (bold line).

listed in Table 1. The precipitation efficiency of amikacin at pH 8.5 was increased by adding ≥1 M equivalent of each aldehyde to amino group, respectively (Fig. 2). However, precipitation efficiency was decreased when pH was raised above 9. Among the tested aldehydes, *trans*-cinnamaldehyde showed the highest precipitation efficacy and was therefore utilized for further experiments. By increasing the molar ratio of amikacin to *trans*-cinnamaldehyde from 1:1 up to 1:5, the percentage of precipitation was enhanced significantly ($p < 0.05$). Employing 1:5 M ratio of amikacin to *trans*-cinnamaldehyde caused 98.8% precipitation of the formed conjugate as illustrated in Fig. 2. Amikacin- *trans*-2, *cis*-6-nonadienal and amikacin-citral conjugates showed similar precipitation efficiency when ratios of 1:4 and 1:5 were tested. Increasing the molar ratio of aldehydes up to 1:4 enhanced significantly ($p < 0.05$) the percentage of precipitation. On contrary, amikacin did not show any precipitation with benzaldehyde in any tested molar ratio. This observation might be explained by the lower lipophilicity of benzaldehyde (log P = 1.69) compared to the other aldehydes (Table 1). Moreover, experiments showed that aldehydes of linear structure are comparatively less efficient, as precipitation efficiency did not exceed 80%. The thermodynamic equilibrium between the educts and the products was dependent on solvents, concentration, pH and temperature [7] as well as steric and electrostatic factors. Since the reaction is reversible, it can go in both directions [17]. The conjugation between amikacin and *trans*-cinnamaldehyde, *trans*-2, *cis*-6-nonadienal and citral was confirmed by the comparison of IR spectra of imine conjugates with that of unmodified amikacin (Figs. 3a–3c). Comparison of the IR spectra of amikacin-aldehyde conjugates (bold line) with the unmodified amikacin (broken line) and aldehydes (dotted lines) showed significant differences in the fingerprint region and confirmed the derivatization. In Fig. 3a at 1634 cm^{-1} there is a band in the spectra of amikacin-*trans*-cinnamaldehyde conjugate (bold line) compared to long appearance at 1669 cm^{-1} for *trans*-cinnamaldehyde alone (dotted line). The same observation can be made in Figs. 3b and 3c. In Fig. 3b there is a band at 1654 cm^{-1} for amikacin-*trans*-2, *cis*-6-nonadienal conjugate (bold line) compared to

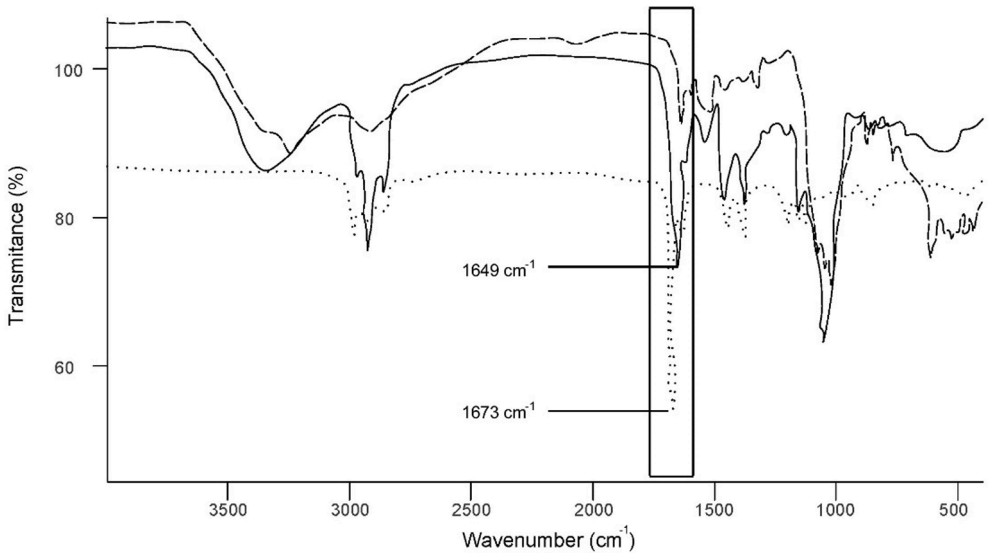


Fig. 3c. Infrared spectrum of amikacin (broken line), citral (dotted line) and amikacin-citral conjugate formed in molar ratio 1:4 (bold line).

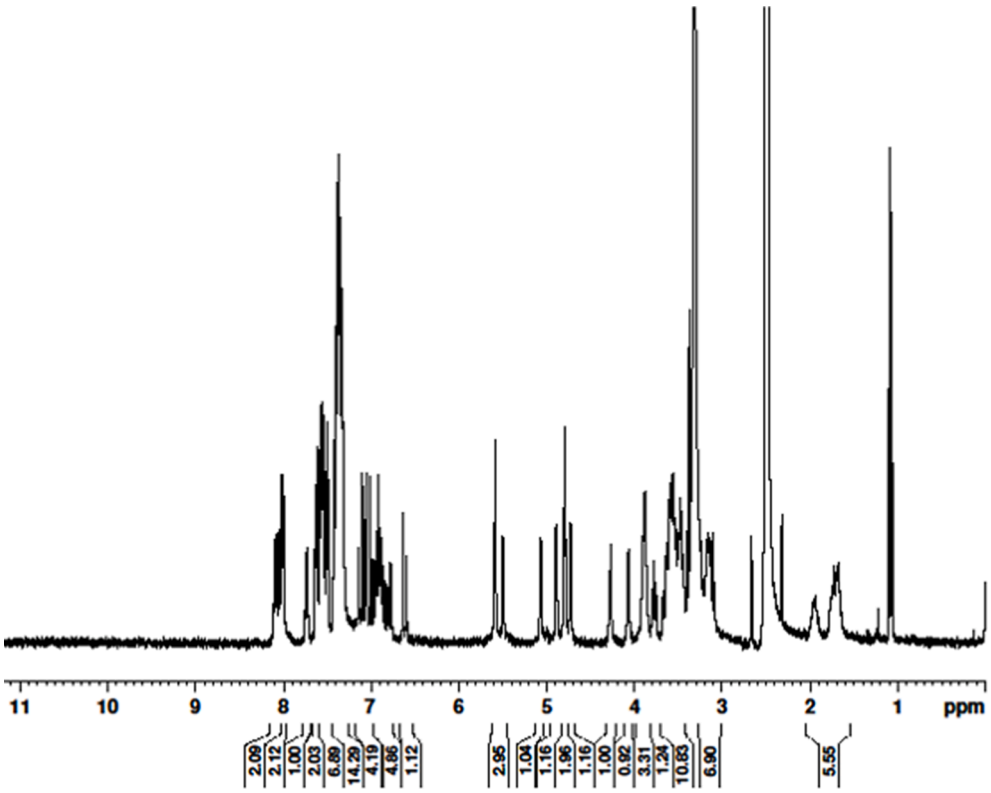


Fig. 3d. ^{1}HNMR spectrum of amikacin-*trans*-cinnamaldehyde conjugate.

long appearance at 1687 cm^{-1} for *trans*-2, *cis*-6-nonadienal (dotted line). In Fig. 3c there is a band at 1649 cm^{-1} for amikacin-citral conjugate (bold line) compared to citral alone (dotted line) showing long appearance at 1673 cm^{-1}.

The formation of the amikacin-*trans*-cinnamaldehyde conjugate was additionally confirmed via ^{1}HNMR analysis. The ^{1}HNMR spectrum of amikacin-*trans*-cinnamaldehyde showed peaks of amikacin protons exchanging with those of cinnamaldehyde as follows (Fig. 3d): 8.09 (d, J = 8.8 Hz, 1H, CH =), 8.05 (d, J = 8.8 Hz, 1H, CH =), 8.02–7.99 (m, 2H, after D_2O exchange: d at 7.99, J = 8.4 Hz, 1H, CH=, C(O)NH), 7.73 (d, J = 8.4 Hz, 1H, CH =), 7.63–7.49 (m, 9H, 8H after D_2O exchange), 7.43–7.31 (m, 14H, 12H after D_2O exchange), 7.14–7.00 (m, 4H), 6.97–6.77 (m, 5H, 4H after D_2O exchange), 6.61 (d, J = 16 Hz, 1H, CH = CH), 5.59–5.58 (m, 2H, D_2O exchangeable), 5.49 (d, J = 5.6 Hz, 1H, D_2O exchangeable), 5.07 (d, J = 2.8 Hz, 1H), 4.89 (d, J = 4.8 Hz, 1H, D_2O exchangeable), 4.80–4.79 (m, 2H, after D_2O exchange: d at 4.79, J = 4.0 Hz, 1H), 4.73 (d, J = 6.8 Hz, 1H), 4.27 (t, J = 5.8 Hz, 1H, D_2O exchangeable), 4.07 (d, J = 7.2 Hz, 1H, D_2O exchangeable), 3.93–3.86 (m, 3H), 3.77 (t, J = 9.2 Hz, 1H), 3.67–3.41 (m, 11*H*), 3.16–3.08 (m, 7H), 1.98–1.63 (m, 6H).

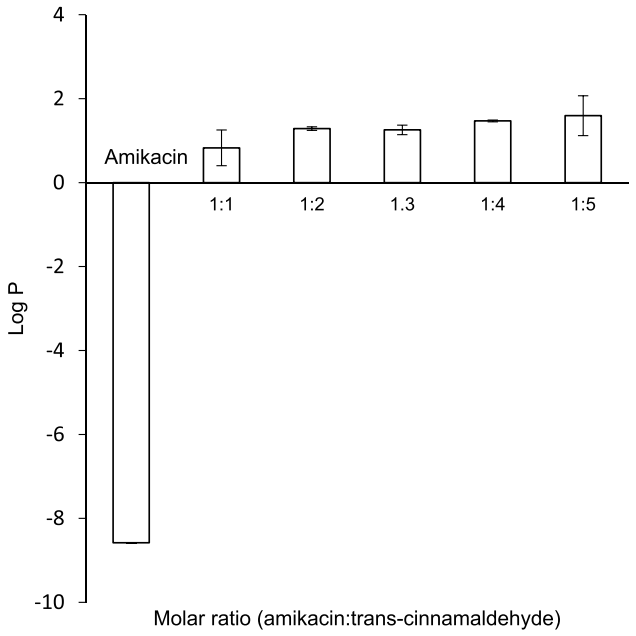


Fig. 4. Log P (partition coefficient) of amikacin and amikacin-*trans*-cinnamaldehyde conjugates in n-octanol/water mixture. The log P of amikacin is a literature value (chemicalize.org). Indicated values are means of at least three experiments ± SD.

4.2. Log P determination

The partition coefficient is the most widely used value to measure lipophilicity. It has an impact on parameters such as permeability [18], solubility [19] and serum albumin binding [20,21]. To confirm the increase in lipophilicity, log P of unmodified amikacin was compared to the log P of formed amikacin-*trans*-cinnamaldehyde conjugates. The investigation was assessed in the lipophilic vehicle n-octanol, whereby the diffusion of the conjugate into the lipophilic phase was demonstrated. Results as shown in Fig. 4 demonstrate an increase in lipophilicity from −8.58 to 0.82 when the conjugate was formed in a molar ratio of 1:1. Increasing the molar ratio of amikacin to *trans*-cinnamaldehyde above 1:4, log P was even further raised reaching its maximum of 1.59 at 1:5 (Fig. 4). This corresponds to an even $1.48*10^{10}$ – fold increase in lipophilicity in comparison to unmodified amikacin. Obtained results are in good agreement with precipitation efficiency results (Fig. 2).

4.3. Release of free amikacin from amikacin-trans-cinnamaldehyde conjugate

To simulate drug release from conjugates at the target site, amikacin-*trans*-cinnamaldehyde was incubated with a model protein at pH 6.8. Since most of the drug will already be released in the systemic circulation on the way to its target site, BSA was chosen as model protein mimicking also human serum albumin, the most prominent plasma protein reaching concentrations of 40 mg/mL (0.6 mM) [20]. Due to a lack of UV chromophores on amikacin, released amikacin could not be detected via this method. Therefore, released cinnamaldehyde from the conjugate along with BSA-*trans*-cinnamaldehyde conjugate were detected, being formed by incubation of amikacin-*trans*-cinnamaldehyde with BSA as displayed in Figs. 5a and 5b. Indeed, the release of amikacin from the conjugate can be explained by the hydrolysis of carbon-nitrogen double bonds, whereby the main step of hydrolysis is the initial attack of water or hydroxide ions [22]. Thus, the dissociation of the conjugate at pH 6.8 could initiate the formation of the new imine conjugates with BSA. Alternatively, amikacin might be simply pushed out of the conjugate by an exchange reaction.

4.4. Formulation and characterization of SEDDS

Development of SEDDS pre-concentrates was based on already published studies with minor modifications [23,24,25]. Components of SEDDS formulations were chosen regarding their suitability to dissolve the amikacin-cinnamaldehyde conjugate, emulsifying properties, resulting droplet size and polydispersity index (PDI). Results of solubility studies are shown in Table 2. Based on the outcome of these solubility studies blank nanoemulsions were prepared and characterized regarding mean droplet size and PDI. As smaller droplets can more easily permeate the mucus gel layer [26], a smaller droplet size was favoured. Formulations that showed no emulsification or an emulsification time >5 min were excluded from further studies (Table 3). Blank nanoemulsions A, E, H and J displayed a mean droplet size ranging from 85.2 to 229.8 nm after 4 h of mild agitation and a PDI ranging from 0.269 to 0.484. Hence, amikacin-*trans*-cinnamaldehyde conjugate was incorporated in these formulations as shown in Table 3. After 4 h of emulsification mean droplet size and PDI of loaded nanoemulsions A, E and H containing 1% conjugate were higher than that of blank formulations. As formulation J with a payload of 1% showed a preferred mean droplet size of 61.5 nm as well as a preferred PDI value of 0.265 it was chosen for further studies. Excipients used for SEDDS pre-concentrates development were oils, surfactants and co-solvents providing a translucent to clear dispersion. In order to solubilize the conjugate Capmul MCM and

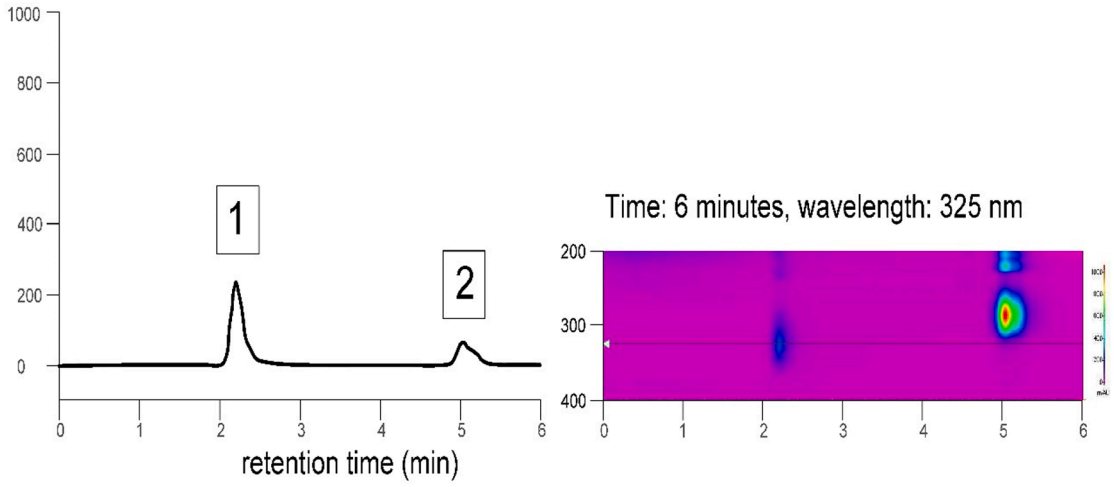


Fig. 5a. Typical HPLC chromatogram with DAD overview of formed amikacin-*trans*-cinnamaldehyde conjugate (peak 1) and *trans*-cinnamaldehyde (peak 2).

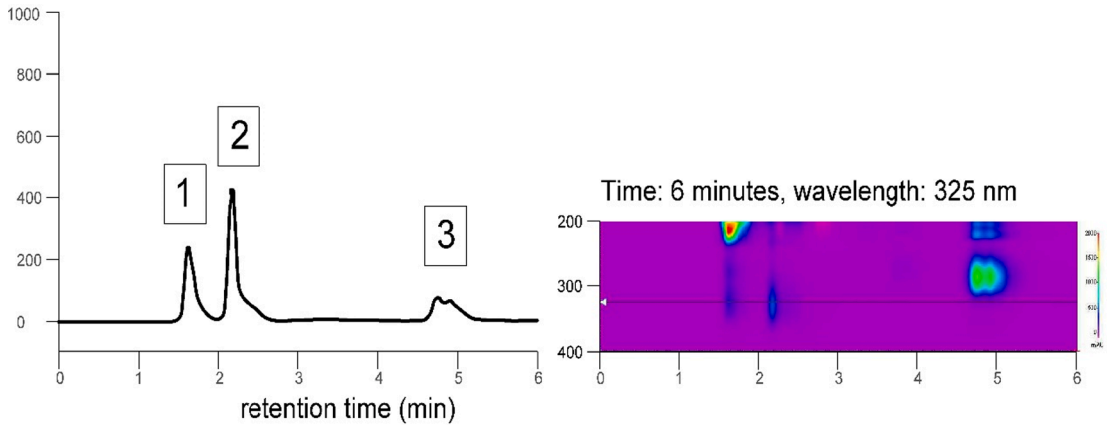


Fig. 5b. Typical HPLC chromatogram with DAD overview of formed bovine serum albumin-*trans*-cinnamaldehyde conjugate (peak 1), amikacin-*trans*-cinnamaldehyde conjugate (peak 2) and *trans*-cinnamaldehyde (peak 3) analysed in DAD, 325 nm wavelength.

Table 2
Solvents used for the development of SEDDS formulation loaded with amikacin-*trans*-cinnamaldehyde conjugate formed in molar ratio 1:5.

Comercial name	Chemical name	HLB	Solubility*
Propylene glycol	Propane-1,2-diol	–	Yes
Mygliol 840	Propylene glycol tricaprylate/ tricaprate	<1	No
Peceol	Glycerol monooleate	1	Yes
Capmul MCM EP	Caprylic/capric mono- and diglycerides	5.5	Yes
Labrafil M 1944 CS	Oleoyl macrogol-6 glycerides	9	Yes
Labrasol	Caprylocaproyl polyoxyl-8 glycerides	12	Yes
Cremophor EL	Macrogolglycerol hydroxystearate	12–14	Yes
Tween 80	Sorbitan monooleate	15	Yes
Tween 20	Sorbitan monolaurate	17	No

*1 mg of amikacin-*trans*-cinnamaldehyde conjugate was randomly dispersed in 20 mg of various lipophilic media as given in Table 2.

Peceol were chosen as oily components. The solubility of the conjugates in Peceol (HLB = 1) and Capmul MCM (HLB = 5.5) indicated that these conjugates tend to be soluble in oils. Peceol (glyceryl monooleate) contains a long chain fatty acid, and Capmul MCM is based on mono- and diglycerides of medium chain fatty acids (mainly caprylic and capric) [1,27]. SEDDS development is mostly dependent on the hydrophilic-lipophilic-balance (HLB) of the surfactant used in these systems. Generally, if a surfactant has a low HLB it will tend to be soluble in oil and a surfactant with a high HLB will tend to be soluble in water [28]. HLB values for excipients used in this publication are literature values [11,29]. Surfactants used herein were Labrafil M1944 CS (HLB = 9), Labrasol (HLB = 12), Cremophor EL (HLB = 12–14) and Tween 80 (HLB = 15). Amikacin-*trans*-cinnamaldehyde conjugate formed in molar ratio 1:5 showed no solubility in highly lipophilic solvents such as Mygliol 840 (HLB < 1) and highly hydrophilic surfactants such as Tween 20 with HLB of 17.

4.5. Log D

As discussed previously [30], the mechanism of drug release from SEDDS is based on a diffusion process from a lipophilic liquid phase into an aqueous liquid phase, whereby the distribution coefficient log D describes the distribution of a sample between a release medium and an oily phase. Due to the submicron size of the droplets, drugs are diffusing very fast from these systems in the release medium reaching equilibrium between SEDDS and the release medium within a few seconds. The drug release from SEDDS is also controlled by the absorption rate of the mucosa, correlating with the concentration of the drug on the membrane and its apparent membrane permeability coefficient (P_{app}). As the formed imine bonds between amikacin and cinnamaldehyde are unstable under acidic conditions, an enteric coating of the final dosageform will be required. The drug can consequently be released just into the intestinal fluid. Log $D_{SEDDS/release\ medium}$ of amikacin-*trans*-cinnamaldehyde conjugate was determined to be 3.38 by the solubility of the conjugate in the SEDDS pre-concentrates and in the release medium [30]. Assuming that 1 mL of the SEDDS pre-concentrate is diluted in 50 mL of intestinal fluid, 4% of the drug would be released immediately. This minor amount of drug being released into the intestinal fluid might be subject to a presystemic metabolism by digestive enzymes. Most of the drug, however, is protected towards such a degradation process, as these enzymes cannot penetrate the oil droplets because of their hydrophilic nature [31]. As reported previously, a log D < 3 will cause an immediate release of a high amount of the drug from SEDDS. A log D > 5 will keep the drug too long in the oily phase to be effectively absorbed by the intestinal mucosa. In this case, the drug release can be accelerated by making use of lipases to degrade the oily droplets [30]. Hence, log D > 3 seems advantageous to enhance drug uptake.

4.6. Cell viability

Viability of Caco-2 cells in the presence of formulations J and H was determined by resazurin assay. Fig. 6 illustrates the toxicity profile of these formulations showing a cell viability over 95% after incubation at 37 °C for 4 h. Caco-2 cells with only HBSS were used as negative control, while cells with 0.1% Triton X-100 in HBSS were used as positive control. SEDDS formulations in applied concentrations seem to have very good tolerability on cells. Results showed a significantly higher cell viability than the positive control. There were no differences in cell viability between the test formulation H and J at significance interval $p < 0.05$.

5. Conclusion

Within this study, imine bond formation turned out as powerful tool for increasing the lipophilicity of the aminoglycoside antibiotic amikacin. Comparing four different aldehydes, the highest precipitation efficiency was reached with *trans*-cinnamaldehyde by utilizing a molar ratio of 1:5. Conjugation with *trans*-cinnamaldehyde resulted in $1.48*10^{10}$ fold increase in lipophilicity of amikacin, indicating a log scale increase from −8.58 to 1.59. Release of amikacin from the amikacin-*trans*-cinnamaldehyde conjugate in the presence of BSA was proven via HPLC. Amikacin-*trans*-cinnamaldehyde conjugate was incorporated into different SEDDS, where very good tolerability of the SEDDS formulations was confirmed by testing on Caco-2 cells for an exposure time of 4

Table 3
Formulation and characterization of basic characteristics of developed SEDDS[a]. Mean droplet size and PDI of each formulation were measured in phosphate buffer after 0 h and after 4 h under mild agitation (37 °C, 300 rpm). Data are shown as mean ± SD (n = 3).

Formulation	Co-solvent	Surfactant				Oily component		Payload (%)	Mean droplet size (nm) ± SD at 0 h	PDI ± SD	Mean droplet size (nm) ± SD at 4 h	PDI ± SD	Emulsification
	Propylene glycol	**Cremophor EL**	**Labrafil M1944 CS**	**Labrasol**	**TWEEN80**	**Capmul MCM**	**Peceol**						
A	30%	45%	–	–	–	25%	–	blank	212.4 ± 5.3	0.515 ± 0.023	157.7 ± 2.7	0.340 ± 0.041	Yes
								1%	224.1 ± 2.7	0.511 ± 0.030	283.6 ± 3.9	0.531 ± 0.032	Yes
B	30%	25%	20%	–	–	25%	–	–	–	–	–	–	No
C	20%	35%	10%	–	–	35%	–	blank	239.8 ± 13.7	0.326 ± 0.029	359.1 ± 39.2	0.526 ± 0.006	Yes
D	25%	20%	–	–	25%	25%	5%	blank	209.1 ± 3.5	0.486 ± 0.032	242.6 ± 4.2	0.476 ± 0.025	Yes
E	20%	20%	–	20%	10%	30%	–	blank	229.2 ± 35.9	0.468 ± 0.017	229.8 ± 71.9	0.441 ± 0.005	Yes
								1%	334.7 ± 14.1	0.578 ± 0.168	364.7 ± 21.0	0.513 ± 0.051	Yes
F	20%	40%	–	–	10%	30%	–	blank	289.0 ± 7.4	0.575 ± 0.023	244.8 ± 0.6	0.460 ± 0.005	Yes
G	20%	25%	–	–	25%	30%	–	blank	283.0 ± 8.7	0.536 ± 0.031	203.2 ± 4.7	0.368 ± 0.052	Yes
H	30%	10%	–	–	40%	–	20%	blank	159.8 ± 14.5	0.579 ± 0.134	143.3 ± 1.2	0.484 ± 0.003	Yes
								1%	243.1 ± 3.1	0.469 ± 0.027	259.8 ± 9.4	0.495 ± 0.133	Yes
I	30%	50%	–	–	–	–	20%	blank	506.3 ± 14.7	0.547 ± 0.038	409.0 ± 9.2	0.530 ± 0.016	Slowly
J	30%	50%	–	–	–	20%	–	blank	80.7 ± 4.3	0.553 ± 0.057	85.2 ± 0.5	0.269 ± 0.006	Yes
								1%	47.3 ± 0.2	0.379 ± 0.018	61.4 ± 1.0	0.265 ± 0.028	Yes
K	20%	55%	–	–	–	10%	15%	blank	209.8 ± 14.8	0.577 ± 0.15	182.2 ± 1.9	0.482 ± 0.004	Slowly
L	20%	60%	–	–	–	10%	10%	blank	133.7 ± 3.2	0.508 ± 0.023	163.8 ± 1.0	0.747 ± 0.010	Slowly
M	20%	60%	–	–	–	–	20%	–	–	–	–	–	No
N	20%	–	–	–	60%	–	20%	blank	195.8 ± 11.2	0.563 ± 0.113	159.1 ± 0.3	0.390 ± 0.007	Slowly
O	30%	–	–	–	50%	–	20%	blank	279.2 ± 8.8	0.445 ± 0.009	236.5 ± 4.1	0.444 ± 0.005	Slowly
P	20%	–	–	60%	–	–	20%	blank	>1000.0	>1.000	>1000.0	>1.000	Yes
Q	30%	50%	–	–	–	10%	10%	blank	169.4 ± 3.7	0.667 ± 0.045	140.0 ± 4.1	0.432 ± 0.063	Yes

[a] SEDDS were prepared by shaking at 25 °C and 1000 rpm for 12 h.

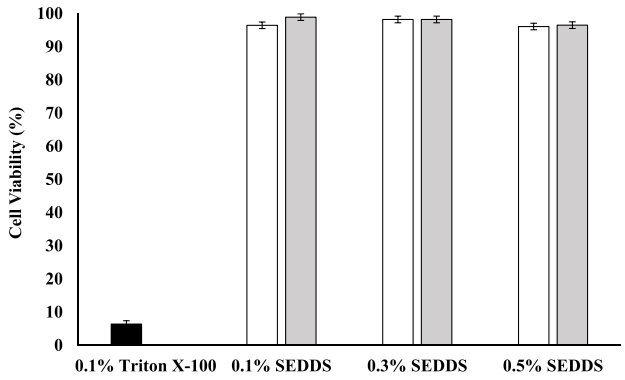


Fig. 6. Cell viability for drug loaded (1% w/w) J and H SEDDS, examined in 0.1%, 0.3% and 0.5% (v/v) emulsions in HBSS where formulation J is presented by white bars, formulation H by light grey bars and positive control by black bars. Data are shown as mean ± SD (n = 3).

h. According to these results, imine bond formation of aminoglycoside antibiotics with *trans*-cinnamaldehyde seems to be a promising strategy for their oral delivery.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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