Development and In Vitro Characterization of Transferrin-Decorated Nanoemulsion Utilizing Hydrophobic Ion Pairing for Targeted Cellular Uptake

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Accepted: 8 March 2021 / Published online: 17 March 2021
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Abstract

Purpose The aim of this study was to develop transferrin-conjugated nanoemulsions utilizing hydrophobic ion pairing for a targeted cellular uptake.

Methods In the lipophilic phase of nanoemulsion composed of 60% oleic acid, 30% Capmul MCM EP and 10% Span 85, 1% cetyltrimethylammonium bromide (CTAB) and 3% phosphatidic acid (PA) were incorporated. After emulsification, the resulting droplets were decorated with human protein transferrin via hydrophobic ion pairing with PA and characterized regarding droplet size and zeta potential. Subsequently, cellular uptake of transferrin-conjugated nanoemulsion was investigated on Caco-2 and HeLa cell lines and determined by flow cytometry, cell lysis method and live cell imaging using confocal laser scanning microscopy.

Results The nanoemulsion showed a droplet size of 123.03 ± 2.1 nm and zeta potential of -54.5 mV that changed because of the surface decoration with transferrin to 182.7 ± 0.2 and $+30.2$ mV, respectively. Within the uptake studies utilizing flow cytometry, transferrin-conjugated nanoemulsion showed a 5.2-fold higher uptake in Caco-2 cells and twofold improvement in case of HeLa cells compared with unmodified formulation. The outcome was confirmed visually via live cell imaging.

Conclusion According to the results, transferrin-conjugated nanoemulsion might be considered as a promising drug delivery system for a selective receptor-mediated drug delivery.

Keywords Nanoemulsion · Hydrophobic ion pairing · Transferrin · Receptor-mediated cellular uptake

Introduction

Effective systemic administration remains for many drugs a challenge due to low bioavailability and selectivity being accompanied by higher costs and toxic side effects. These challenges could be effectively addressed with innovative drug delivery systems. Among them, lipid-based drug delivery systems moved in the limelight of research providing beneficial properties to overcome physiological barriers such as mucus gel layer and cell membranes [1, 2]. Moreover, additional surface modifications such as decoration with targeting ligands provide more specific and target-orientated

drug delivery [3]. In particular, specific cellular uptake can be enabled by interaction between a targeting ligand and the corresponding receptor being located on the cellular surface. Among such targeting ligands, the serum glycoprotein transferrin, which transports iron in the body and internalizes it into cells via receptor-mediated endocytosis, is of particular interest [4]. As the transferrin receptor is overexpressed to a higher degree on rapidly proliferating cells, whereas in case of non-proliferating cells, its expression is comparatively low; it could be used to improve selective cellular uptake of free drugs and nanocarriers by diseased tissue [5, 6]. An effective receptor-mediated internalization of transferrin-conjugated anticancer drugs as well as nanocarriers such as liposomes and nanoparticles, for instance, has already been demonstrated in several studies [7–11].

A common method to decorate nanocarriers with transferrin is based on the covalent attachment of this protein on their surface [12–15] although this chemical modification might have an impact on protein conformation and efficacy [7, 8].

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Moreover, the chemical modification of well-established auxiliary agents causes from the regulatory point of view a huge difference as these auxiliary agents are then regarded as new entity that needs to go through the full toxicity program and registration process such as a new active pharmaceutical ingredient. In the present study, the droplet surface of a nanoemulsion was modified with transferrin via hydrophobic ion pairing. In contrast to all other so far utilized methods for surface decoration of lipid-based nanocarriers with transferrin, this method is based on just non-covalent ionic interactions between oppositely charged functional groups of the drug and a surfactant [16]. The potential of nanoemulsions displaying transferrin on their surface just via hydrophobic ion pairing on cellular uptake by Caco-2 and HeLa cells utilizing various methods such as flow cytometry, live cell imaging and cell lysis method is investigated.

Materials and Methods

Materials

Cetyltrimethylammonium bromide (CTAB), oleic acid, Span 85 (sorbitan trioleate, HLB = 1.8) and transferrin human were purchased from Sigma-Aldrich (Austria). Transferrin human CF® Dye Conjugate (Cf594) was purchased from Biotium, Inc. (USA). 1,2-Dipalmitoyl-sn-glycero-3-phosphatidic acid disodium salt (PA) was purchased from Bachem Holding AG (Switzerland). Lumogen F Yellow 083 was a gift from BASF (Germany). Hoechst 33528 was purchased from Thermo Fisher Scientific (USA). All other chemicals, reagents and solvents were of analytical grade and received from commercial sources. Other cell culture supplies were purchased from Biochrom GmbH (Germany).

Hydrophobic Ion Pairing

Within this study, the ability of transferrin (Tf) to form complexes with oppositely charged surfactants via hydrophobic ion pairing (HIP) was investigated. HIP was performed according to a method described by Zupančič et al. with minor modifications [17]. Therefore, anionic PA and cationic CTAB surfactants were investigated with respect to their suitability as ion pairing partner utilizing molar ratios from 10:1 to 300:1 of surfactant to drug. A total of 200 µL of anionic surfactant solution of PA and cationic surfactant solution of CTAB (10–300 µM), respectively, were added slowly and dropwise to 200 µL of Tf solution (1 µM) under gentle stirring at 300 rpm (Eppendorf ThermoMixer® C, Hamburg, Germany) at room temperature. In case of complex formation with PA, pH of Tf solution was adjusted to pH 2 with 0.1 M HCl and in case of CTAB to pH 10.5 with 0.2 M NaOH. The mixtures of Tf-surfactants

were incubated for 1 h, and subsequently, the precipitated Tf-surfactant complex was isolated by centrifugation (5 min, 10,000 rpm) (Eppendorf® Minispin®, Hamburg, Germany). The precipitation efficiency of Tf-surfactant complex was calculated by quantification of the remaining amount of Tf in the supernatant by Micro BCA-protein assay kit (Thermo Fischer Scientific, USA) using Eq. 1:

$$\text{Precipitation efficiency}[\%] = 100 - \left(\frac{C_s}{C_t} \times 100 \right) \quad (1:)$$

where C_s is the Tf concentration in supernatant and C_t is the total Tf concentration.

Nanoemulsion Development and Characterization

Oleic acid (60% (m/m)), Capmul MCM EP (30% (m/m)), and Span 85 (10% (m/m)) were homogenized by a vortex mixer to form a single phase as described previously by Zupancic et al. [18]. Additionally, CTAB (1% (m/m)) and PA (3% (m/m)) were dissolved one after the other in the pre-concentrate followed by homogenization of the formulation at 800 rpm with thermomixer (Eppendorf ThermoMixer® C, Hamburg, Germany) at room temperature for 24 h. Subsequently, the formulation was emulsified in aqueous medium utilizing vortex mixer for 5 min followed by ultrasonication in a glass beaker via Ultrasonic Processor UP200H (Hielscher Ultrasonics GmbH, Teltow, Germany) with 100% amplitude and pulse for 3 min. The formulation was centrifuged at 13,400 rpm and 25 °C for 5 min and visually examined regarding stability of the emulsion. In order to obtain Tf-conjugated droplets, Tf was first dissolved in water previously adjusted to pH 2 with 0.1 M HCl. Subsequently, Tf solution was dropwise added to nanoemulsion (NE) under gentle agitation. The obtained Tf-conjugated droplets were characterized regarding mean droplet size, polydispersity index, and zeta potential by dynamic light scattering using a Zetasizer Nano ZSP (Malvern Instruments, Worcestershire, UK). All measurements were performed at 37 °C. In order to ensure the stability of the NE formulation for further experiments, droplet size and polydispersity index were investigated at 300 rpm stirring and 37 °C over 4 h. Additionally, long-term storage stability studies at 4 °C, 25 °C and 37 °C were conducted over 21 days.

Fluorescence Imaging for Colocalization of Single Droplet and Tf/CF594

First, 0.15% (m/m) Lumogen F Yellow 083 (LGY) was dissolved in oily phase of the formulation at 37 °C under stirring at 1000 rpm using a thermomixer for 12 h. Subsequently, NE was prepared in the same manner as described above utilizing ultrasonication. Tf labelled with CF 594

fluorescent dye was attached to the droplet surface via HIP providing a protein corona on the droplet surface.

Secondly, NE was diluted to the final concentration of 0.025% [v/v] in water and investigated regarding colocalization between LGY incorporated into the oily droplets and Tf/CF594 attached to the droplet surface utilizing confocal laser scanning microscope (SP8, Leica, Germany). LGY-labelled unconjugated NE as well as free Tf/CF594 served as a control.

Viability of Caco-2 and HeLa Cells

The concentration-dependent 0.05–1% (w/v) cytotoxicity of NE and NE-Tf was investigated on Caco-2 cells as well as on HeLa cells using flow cytometry. Flow cytometry cytotoxicity assay is based on propidium iodide (PI) staining of DNA fragments in damaged cell.

Cells were seeded in 24-well plates (Greiner Bio-One, Germany) with an initial concentration of 2.5×10^4 cells/mL and cultivated in Minimum Essential Medium Eagle (MEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) and penicillin/streptomycin solution (100 units/0.1 mg/L) at 37 °C, 95% humidity, under 5% CO₂ atmosphere for 5 days. The cell concentration was determined using the Countess™ automated cell counter (Invitrogen, Korea). Further, cells were washed with 500 µL of preheated 10 mM PBS pH 7.4 twice and incubated with at 37 °C preheated OptiMEM for 30 min. NE was prepared in OptiMEM and subsequently conjugated with Tf. Thereafter, 500 µL of NE and NE-Tf, respectively, were added to the cell layer and incubated for 3 h. Subsequently, cells were washed thrice with 10 mM PBS pH 7.4 and treated with 200 µL of Accutase for 10 min, in order to detach cells from the plate surface followed by re-suspension with ice-cold 10 mM PBS pH 7.4. After re-suspension, cells were treated with PI staining solution for 1 min and immediately analysed by flow cytometry utilizing Attune NxT Flow Cytometer (Thermo Fisher Scientific, USA). Cells were analysed in triplicate for each cell type using excitation wavelength of 488 nm and emission wavelength of > 600 nm, previously gating out a residual debris. Cells treated only with OptiMEM served as negative control and with 1% [m/v] Triton X-100 solution as positive control.

Cellular Uptake

Flow Cytometry

Within this study, cellular uptake of Tf-conjugated NE was investigated on Caco-2 cells as well as on HeLa cells utilizing flow cytometry (Attune NxT Flow Cytometer) following a slightly adopted method described by Friedl et al. [19]. In brief, formulation labelled with 0.15% (m/m) LGY was

conjugated with Tf via HIP building up a protein corona as already described above. Subsequently, Tf-conjugated NE was diluted with OptiMEM to a final concentration of 0.025% (v/v). Cells were seeded in 24-well plates (Greiner Bio-One, Germany) with an initial concentration of 2.5×10^4 cells/mL and cultivated in Minimum Essential Medium Eagle (MEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) and penicillin/streptomycin solution (100 units/0.1 mg/L) at 37 °C, 95% humidity, under 5% CO₂ atmosphere until 100% confluence was reached. Cells were washed twice with prewarmed 10 mM PBS pH 7.4 and incubated with OptiMEM for 30 min. Subsequently, Caco-2 cells as well as HeLa cells were incubated with NE and NE-Tf for 3 h, respectively. Afterwards, cells were washed thrice with 10 mM PBS pH 7.4 and subsequently treated with 200 µL Accutase for 10 min in order to detach the cells from the plate surface, followed by re-suspension with ice-cold 10 mM PBS pH 7.4. Cell suspension was immediately analysed regarding uptake via flow cytometry (Attune NxT Flow Cytometer) using excitation wavelength of 488 nm and emission wavelength of > 600 nm, previously gating out a residual debris. The data were analysed by custom written MatLab program and fluorescence intensity distribution data were shown applying the ‘logicle’ display method developed by Parks et al. [20]. The relative mean fluorescence intensity values (RMFI) quantify the fluorescent intensity of LGY-labelled NE being proportional to their average concentration per cell. RFMI was calculated from the mean fluorescence intensity values (MFI), according to the following Eq. 2:

$$\text{RMFI} = \frac{\text{MFI(S)}}{\text{MFI(C)}} - 1 \quad (2)$$

where RMFI value of the control samples (Caco-2 cells or HeLa cells only) is always zero, MFI(S) represents the mean fluorescence intensity of the treated sample and MFI(C) the mean fluorescence intensity of the control. Results are presented as mean $\pm$ SD, $n = 3$.

Cell Lysis

In order to confirm the outcome of flow cytometry study, cellular uptake was additionally evaluated by cell lysis followed by protein normalization using a slightly modified method adapted from Sahoo et al. [21]. NE was labelled with 0.15% [m/m] LGY followed by conjugation with Tf. Caco-2 cells as well as HeLa cells were cultured in 24-well plates (Greiner Bio-One, Germany) over 5 days with an initial concentration of 2.5×10^4 cells/mL until 100% confluence was reached. Cell layer was incubated with NE, NE-Tf and NE in presence of free Tf for 3 h, respectively. Subsequently, cells were washed thrice with 500 µL of PBS pH

7.4 and incubated with 150 μL of RIPA lysis buffer (Thermo Fischer Scientific, USA) for 30 min. Fluorescence intensity was determined fluorometrically after background subtraction using an excitation wavelength of 488 nm and an emission wavelength of 530 nm with microplate reader (Spark, Tecan, Grödig, Austria). The amount of protein per well was quantified via Micro BCA-protein assay kit (Thermo Fischer Scientific, USA).

Live Cell Imaging

Caco-2 cells and HeLa cells were cultured in μ -Slide 8 well glass bottom sample holder (ibidi, Germany) over 5 days with an initial concentration of 2.5×10^4 cells/mL. Cells were incubated with 200 μL of LGY-labelled formulations NE and NE-Tf, respectively, in a final concentration of 0.025% for 3 h. Subsequently, cells were washed thrice with preheated PBS pH 7.4 and placed onto the microscope stage for further 10 min. The nucleus was stained using Hoechst 33528 (1 $\mu\text{g/mL}$). Images were obtained by confocal laser scanning microscope (SP8 gSTED microscope, Leica, Germany). Image post performed using the open source image processing and analysis platform ImageJ: where the yz- and xz-projections were prepared from 5 xy-images of an image stack taken at 0.2 μm z-step length. Moreover, fluorescence bleed-through between detection channels was deleted applying spectral unmixing. 2D image filtering was conducted using a Gaussian filter.

Statistical Data Analysis

Statistical data analysis was performed on GraphPad Prism (version 5.01) using the Student's *t* test and the analysis of variance (ANOVA) followed by Bonferroni correction with $p < 0.05$ as the minimal level of significance. All values are expressed as means $\pm$ SD.

Results and Discussion

Hydrophobic Ion Pairing

Within this study, the capability of Tf to form HIPs with both a cationic and anionic surfactant was investigated. As demonstrated in Fig. 1, precipitation efficiency of complexes increased with an increase in surfactant to Tf ratio. Human serum Tf is composed of ~ 140 amino acids containing alkaline side chains and ~ 95 amino acids with acidic side chains [22]. In order to mask as many of these binding sites as feasible, a surfactant to Tf molar ratio between ~ 100 and 150 to 1 was chosen. At the molar ratio of 150 to 1, the precipitation efficiency of the complex reached the highest level. In case of anionic surfactant PA, HIP complex precipitated

Fig. 1 Hydrophobic ion pairing of Transferrin (Tf) with anionic surfactant 1,2-Dipalmitoyl-sn-glycero-3-phosphatidic acid (PA) [○] and cationic surfactant cetyltrimethylammonium bromide (CTAB) [■]. After the addition of surfactants, the precipitated Tf complex was centrifuged and the remained Tf in supernatant was analyzed by Micro BCA-protein assay. Data are shown as means ($n \geq 3$) $\pm$ SD

almost completely reaching a precipitation efficiency of nearly 100%, whereas the cationic surfactant CTAB reached a precipitation efficiency of $\sim 70\%$. A further increase of the molar ratio did not show any significant changes in precipitation efficiency. The higher extent of precipitation using PA is likely related to the higher lipophilicity of this anionic surfactant. PA is composed of two C16 alkyl chains resulting in a more lipophilic complex in comparison with CTAB with only one C16 alkyl chain.

Nanoemulsion Development and Characterization

Within this study, a novel nanoemulsion formulation decorated with human Tf was developed and characterized regarding size, PDI, and zeta potential (Table 1). The formulation consisting of 60% oleic acid, 30% Capmul MCM EP and 10% Span 85 followed by additional incorporation of 1% (w/w) CTAB and 3% (w/w) phospholipid PA was used for further studies. The surfactants CTAB and PA were added in order to stabilize the emulsion and to serve as counter ion for pairing with Tf. Phospholipid PA is widely used as emulsion

Table 1 Droplet size, polydispersity index (PDI) and zeta potential of developed formulations NE and NE-Tf

Droplet size [nm]	PDI	Zeta potential [mV]
NE	123.03 $\pm$ 2.12	0.239
NE-Tf	182.70 $\pm$ 0.17	0.184

Fig. 2 The chemical structure of HIP complex between phosphatidic acid incorporated into nanoemulsion and Transferrin

stabilizer and is responsible for a highly negative surface charge and long-term stability under test conditions as described previously [23, 24]. Tf was attached on the droplet surface via HIP providing a protein corona. HIP is based on non-covalent ionic interactions between oppositely charged functional groups of Tf and the surfactant [16]. Negatively charged phosphate groups of PA exhibited ionic interactions with positively charged amine groups of Tf at acidic pH forming a corona on the droplet surface (Fig. 2). Thereby, the droplet size increased up to ~182 nm after Tf attachment. Due to the shielding of the negatively charged phosphate groups by the Tf attachment, zeta potential increased up to 30 mV, confirming that Tf is located on the droplet surface. Stability study showed no change in droplet size of NE and only a minor change of Tf-conjugated NE (NE-Tf) within 24 h under simulated physiological conditions (Table 2). Moreover, no significant difference in droplet size of NE could be observed conducting a long-term storage stability study over 21 days at 4 °C, 25 °C and 37 °C (S1) [25, 26]. Furthermore, the surface modification via HIP is considered as gentle and effective without causing conformation

changes and activity loss of the protein. In contrast, widely used covalent conjugation methods might have an impact on the protein due to the chemical reaction. Wang et al., for instance, performed the modification of the surface of solid lipid nanoparticles with Tf via click chemistry utilizing a carbodiimide-mediated crosslinking [27]. However, this chemical modification might affect the ability of the protein to bind to its receptor and therefore the effectiveness of a receptor-mediated uptake into cells.

Fluorescence Imaging for Colocalization of Single Droplets and Tf/CF594 Conjugate

In order to confirm the localization of Tf on the droplet surface, fluorescence imaging of single droplets and Tf/CF594 conjugate was conducted via confocal laser scanning microscopy. As displayed in Fig. 3, LGY labelled NE decorated with Tf/CF594 (NE-Tf/CF594) demonstrated high fluorescence intensity at the wavelength of 488 nm (LGY channel) as well as at 594 nm (Tf/CF594 channel). Merged image showed both dyes colocalized by each other. As a control, used LGY labelled NE appeared only in LGY channel, showing no fluorescence at the wavelength of 594 nm at all. Blank Tf/CF594 could only be observed at 594 nm as a background. Consequently, the merged image showed the colocalization of Tf and the LGY labelled droplet confirming that Tf molecules are widely attached to the droplet surface exhibiting a protein corona.

Cell Viability of Caco-2 and HeLa Cells

Within this study, cell viability in the concentration range from 0.05 to 1% (w/v) was evaluated on Caco-2 cells and HeLa cells. Results are illustrated in Fig. 4. In terms of Caco-2 cells, the cell viability after treatment with NE at the

Table 2 Droplet size and polydispersity index (PDI) of reconstituted NE and NE-Tf formulations as a function of time. Data are shown as mean $\pm$ SD ($n = 3$)

NE			NE-Tf	
Time [h]	Droplet size [nm]	PDI	Droplet size [nm]	PDI
0	123.03 $\pm$ 2.12	0.239	182.70 $\pm$ 0.17	0.184
1	123.97 $\pm$ 0.91	0.234	182.03 $\pm$ 0.65	0.169
2	124.73 $\pm$ 0.60	0.231	178.80 $\pm$ 1.35	0.109
3	124.87 $\pm$ 1.14	0.236	178.30 $\pm$ 0.72	0.107
4	124.97 $\pm$ 0.97	0.232	174.10 $\pm$ 0.53	0.091
24	125.33 $\pm$ 1.31	0.220	175.23 $\pm$ 1.92	0.104

Fig. 3 Lumogen Yellow (LGY) labelled NE conjugated via HIP with Tf/CF594 (NE-Tf/CF594) demonstrated high fluorescence intensity in LGY channel at 488 nm, in Tf/CF594 channel at 594 nm and colocalization of both dyes in merged image. LGY labelled unconjugated NE appeared only in LGY channel at 488 nm and Tf/CF594 only in Tf/CF594 channel at 594 nm as background

concentration of 0.05% reached over 77% after 3 h of incubation (Fig. 4a). Increasing the concentration up to 0.1% and 1%, respectively, cell viability decreased to ~37% and ~17%, respectively. In contrast, viability of cells after incubation with Tf-decorated NE reached 83% at the concentration of 0.05%, 61% in case of 0.1% and 24% in case of 1% within 3 h.

Cytotoxicity assay conducted at HeLa cells demonstrated comparable results, showing a moderate toxicity with a cell viability of 66% in case of NE and 86% in case of NE-Tf at the concentration of 0.05% (Fig. 4b). However, cell viability at the concentrations of 0.1% and 1% for both NE and Tf-decorated NE decreased below 15%.

Cells treated with blank OptiMEM reached viability over 95%, whereas the treatment with 1% Triton X-100 solution leads to almost 100% cell death.

The cytotoxic effect at higher concentrations might be partly induced due to the cationic surfactant CTAB. As previously reported by Lam et al., cationic surfactants could exhibit cytotoxic effects being incorporated into lipid-based nanoemulsions such as self-emulsifying drug delivery system (SEDDS) [28]. However, used amount of CTAB is crucial for the formulation, providing stability and, where

appropriate, serving as counter ion for HIP. In case of the Tf-decorated formulation, a comparatively lower cytotoxicity might be explained by the protein layer on the droplet surface masking the cationic surfactant and reducing the interaction of it with cell membrane. According to this outcome, low cytotoxicity of the formulations provides safety and biocompatibility with regard to the possible application on cells.

In order to exclude any toxic effects of the formulation on cells, the formulation was used in further studies in a final concentration of 0.025%.

Cellular Uptake

Cellular uptake of the Tf-decorated formulation was carried out by measuring the fluorescence activity of LGY, which was previously incorporated into the formulation. LGY is highly lipophilic fluorescent dye with a logP of 7.4 that remains in the oily phase of the formulation. Uptake was evaluated, conducting flow cytometry and cell lysis studies on human colon adenocarcinoma Caco-2 cells and cervix adenocarcinoma HeLa cells. The outcome of these studies

Fig. 4 Cell viability rates (in [%]) of Caco-2 cells **A** and HeLa cells **B** after 3 h incubation with blank NE (black bars) and Tf-conjugated NE (white bars) at the concentrations of 0.05%, 0.1% and 1%. As a negative control, cell viability was determined after incubation with OptiMEM. 1% [w/v] Triton X-100 solution serves as a positive control. Data are shown as means ($n \geq 3$) $\pm$ SD

was additionally confirmed by live cell imaging via confocal laser scanning microscopy.

The uptake study utilizing flow cytometer demonstrated an improvement of cellular uptake properties of Tf-decorated formulation in comparison with the blank formulation. NE-Tf showed a 5.2-fold higher uptake on Caco-2 cells and twofold improvement in case of HeLa cells compared with NE (Fig. 5a and b).

In order to confirm the results obtained in the uptake study utilizing flow cytometry, further investigations using a cell lysis method were conducted. Within the cell lysis study, NE-Tf provided a significantly higher cellular uptake into Caco-2 cells compared with NE without Tf corona (Fig. 6a). Moreover, the receptor-mediated uptake of Tf-decorated formulation was confirmed additionally investigating the uptake of blank NE in a presence of free Tf. No significant difference in uptake of NE in the presence and absence of free Tf could be observed. This outcome showed that only attached Tf has an impact on the internalization of droplets

into the cell, whereas free Tf does not have any effect on the uptake of nanocarrier at all. In terms of HeLa cells, NE-Tf exhibited similar results in the uptake as obtained on Caco-2 cells. As presented in Fig. 6b, NE-Tf showed a significant difference in comparison with blank NE with and without free Tf.

Finally, live cell imaging via confocal laser scanning microscopy was performed highlighting visually Tf-mediated uptake in comparison with blank formulation. As illustrated in Fig. 7, LGY-labelled Tf-decorated droplets accumulate mainly in the cytosol of Caco-2 cells, whereas only a minor amount could be observed in cell nucleus. In contrast, only a poor uptake of the blank NE into the cells was achieved. Similar results could be observed on HeLa cells. While Tf-decorated formulation NE was superiorly internalized, the blank formulation NE demonstrated a comparatively lower uptake as presented in Fig. 7.

These results provide strong evidence that a covalent attachment of Tf is not a prerequisite in order to

Fig. 5 Cellular uptake of Tf-conjugated NE after 3 h incubation on a Caco-2 **A** and Hela **B** cell lines at a concentration of 0.05 % (v/v) by flow cytometry. Shift in fluorescence intensity in contrast to control (black) is displayed in case of NE in red and in case of NE-Tf

in blue. Relative mean fluorescence intensity values (RMFI) quantify the fluorescent intensity of LGY-labelled nanoemulsion droplets per cell. (***) $p < 0.001$

improve the cellular uptake of lipid-based nanocarriers. According to our results, an ionic attachment of Tf on the surface of nanocarriers is sufficient to guarantee cell binding and internalization. Utilizing Tf-decorated formulation NE, various compounds such as anticancer drugs, anti-inflammatory drugs, therapeutic peptides, and even therapeutic genes could be efficiently and selectively delivered to their target cells via receptor-mediated endocytosis [4]. Transferrin receptor (TfR)-mediated endocytosis provides a selective and effective uptake into different cancer cell lines. In order to meet the increased need of iron, diseased cells, such as proliferated malignant cells, overexpress TfR, thereby internalizing a higher amount of Tf [29]. Moreover, recent studies showed TfR overexpression not only on proliferated malignant cells but also on the cell surface exposed to pro-inflammatory factors. Harel et al. investigated TfR expression on the colon epithelium cells of the patients

with inflammatory bowel diseases (IBDs) and accomplished enhanced targeting of these cells by liposomes decorated with anti TfR [5]. Several studies have already demonstrated an effective and selective targeting mediated by TfR [7, 8]. Guo et al. investigated cellular uptake of Tf-conjugated doxorubicin-loaded lipid coated nanoparticles showing enhanced targeting of lung cancer cells [8]. This outcome is also in a good agreement with results presented by Anabousi et al. demonstrating an enhanced uptake level of Tf-conjugated liposomes at different cell lines [7].

As reported in prior studies, nanoemulsions provide a great potential in drug delivery, showing enhanced mucus permeating properties as well as the ability to be internalized into cells. Griesser et al., for instance, investigated mucus permeating properties of self-emulsifying drug delivery systems, comparing them with other nanocarriers such as nanoparticles and liposomes, showing with a

Fig. 6 Cellular uptake of Tf-conjugated NE, presented as fluorescence intensity per μg protein, in Caco-2 **A** and HeLa **B** cell lines utilizing the cell lysis method. Cells incubated with NE in absence (NE) or presence (NE/Tf) of free Transferrin served as control. Data are shown as means ($n \geq 3$) $\pm$ SD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in comparison to NE-Tf)

twofold improvement the advantage of self-emulsifying drug delivery systems [1]. Hauptstein et al. incorporated pDNA into the oily phase of a nanoemulsion and demonstrated an improved cellular uptake due to this delivery system [2]. Moreover, oral bioavailability of cancer drugs such as paclitaxel, 5-fluorouracil or piplartine was enhanced utilizing nanoemulsions [30–32]. Yang et al. investigated pharmacokinetics of paclitaxel containing self-microemulsifying drug delivery systems showing in an in vivo study an enhanced oral bioavailability [30].

Within this study, the performance of nanoemulsions was further improved by Tf decoration providing enhanced cellular uptake. In contrast to so far established lipid-based nanocarriers having been decorated with Tf via covalent attachment, we could demonstrate within this study that the same effect of Tf can be achieved by making use of simple ionic interactions. This new approach might be also applicable for numerous other ligands avoiding the use of new chemical entities such as targeting ligand-surfactant conjugates that would be subject of an entire registration process.

Fig. 7 Cellular uptake of blank NE and Tf-conjugated NE into Caco-2 and HeLa cell lines visualized by confocal microscopy. Nuclei were stained with Hoechst 33528 and NE-Tf was labeled with Lumogen Yellow

Conclusion

It was the aim of this study to modify the surface of the oily droplets of a nanoemulsion with human Tf via ion pairing and to provide evidence for the efficacy of this approach. A simple but effective method for surface decoration with a HIP complex between the targeting ligand and charged surfactants was established. As demonstrated by flow cytometry, cell lysis studies and live cell imaging on Caco-2 cells as well as on HeLa cells, Tf-receptor-mediated endocytosis was significantly improved by a stable Tf-corona formed on the surface of the oily droplets. These results confirm the great potential of Tf-decorated nanocarriers regarding an improved and targeted cellular uptake. Tf-decorated nanoemulsions might be considered as promising drug delivery systems for selective receptor-mediated internalization of various drugs. Moreover, the concept of ionic attachment of Tf to the surface of lipid-based nanocarriers might be transferred to other targeting ligands.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12247-021-09549-2>.

Acknowledgements This work was supported by a doctoral scholarship for the promotion of young researchers at the Leopold-Franzens-University Innsbruck.

Funding Open access funding provided by University of Innsbruck and Medical University of Innsbruck.

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