2021

Peptide Conjugation to Vitamin K loaded Mixed Micelles

MAJOR RESEARCH PROJECT

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Layman's Summary

Vitamin K is a cofactor for an enzyme that plays a role in blood coagulation. When vitamin K concentrations are too low in breastmilk, infants will not receive enough vitamin K, possibly leading to vitamin K deficiency bleeding. This can cause internal bleedings, neurological damage or death. Infants can receive vitamin K through medication in order to treat and prevent vitamin K deficiency. A formulation to provide this is Konakion® mixed micelles Paediatric. This formulation can be administered orally, meaning that the drug has to travel via the stomach to the intestine, where it can be absorbed through cells called enterocytes. After absorption, vitamin K will enter the lymphatic system and subsequentially the blood circulation.

Vitamin K is lipophilic, which is why it is transported in the core of a micelle. This micelle has hydrophilic heads on the surface and hydrophobic tails pointed to the core, making the core of the micelle suited for vitamin K. When the drug is administered, the micelles enter the stomach, where the pH is low. This can lead to aggregation of the micelles. When the micelles leave the stomach and enter the intestine, bile salts help to solubilize these aggregates, so that the uptake of the micelles by enterocytes can proceed. However, some infants have a condition called cholestasis, involving that bile salts are not sufficiently produced. In case of cholestasis, the intestinal uptake of the micelles is low.

To improve the stability in the stomach and increase the uptake in the intestine, modifications can be made to the existing Konakion® mixed micelles Peadiatric. Research has shown that adding a component called polyethylene glycol to the surface of the mixed micelles forms a steric barrier. This barrier increases the stability at a low pH. In this project, we examined if a peptide could be bound to the surface of the micelle to increase the uptake in the intestine. R4F is a peptide that has a high affinity to receptor SR-B1, located in the enterocytes. The peptide cRGD is an integrin binding motif that binds to integrin αVβ3, located on intestinal cells called M-cells. Addition of these peptides could lead to increased affinity between the cells in the intestine and the micelles, which could lead to increased uptake. When the uptake is increased, more vitamin K will end in the blood circulation. In this project, R4F and cRGD were separately conjugated to the micelles. To determine if the peptide was successfully bound to the micelle, various aspects were measured such as size, charge, mass and the peptide concentration in buffers that were added to the micelles. When those tests were successful, human cells expressing SR-B1 or integrin αVβ3 were incubated with micelles that were not conjugated to a peptide, and with micelles that were conjugated to R4F or cRGD. Because the cell nuclei, SR-B1 receptors and the micelles were stained with a fluorescent label using different colors, it could be determined if the peptide conjugated micelles had an increased binding and uptake by SR-B1 receptors, compared to the micelles without a peptide conjugate.

Abbreviations

ACN	Acetonitrile
ApoA1	Apolipoprotein A1
CMS	Chylomicrons
cRGD	Cyclic arginyl-glycyl-aspartic acid
Cys	Cysteine
DCM	Dichloromethane
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle's Medium
DMF	Dimethylformamide
DSPE-PEG	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene-glycol)
EDTA	Ethylenediaminetetraacetic acid
EPC	Egg phosphatidylcholine
ESI	Electrospray ionization
GFP	Green fluorescence protein
HDL	High-density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
MALDI TOF	Matrix-Assisted Laser Desorption Ionization–Time of Flight
MS	Mass Spectrometry
NPs	Nanoparticles
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
R4F	Reverse-4F
RGD	Arginyl-glycyl-aspartic acid
rHDL	Reconstructed HDL
SR-B1	Scavenger receptor class B type 1
TFA	Trifluoroacetic acid
UPLC	Ultra performance liquid chromatography
UV	Ultra violet
VKDB	Vitamin K Deficiency Bleeding

Abstract

Vitamin K_1 functions as a cofactor for γ-glutamyl carboxylase, an enzyme affecting various blood coagulation factors. Vitamin K deficiency is often present in neonates due to limited transport through breast milk, possibly leading to vitamin K deficiency bleeding (VKDB). Uptake of vitamin K is predominantly performed by scavenger receptor class B type 1 (SR-B1) on enterocytes. SR-B1 mediates transport of lipids, predominantly of cholesterol esters carried by high-density lipoprotein (HDL). In the intestine, metabolism of vitamin K is facilitated by bile salts. In case of cholestasis, limited amounts of bile salts are released into the intestine, causing malabsorption of vitamin K. A commercial and clinically used vitamin K formulation for oral delivery, is Konakion® mixed micelles Paediatric. This formulation contains egg phosphatidylcholine (EPC) or lecithin, glycocholic acid and vitamin K_1. Exposure of the formulation to gastric acid leads to protonation of the carboxylic acid group of glycocholic acid. This causes large aggregates to arise and enter the intestine. Bile salts solubilize these aggregates, however in case of cholestasis aggregates remain insolubilized, leading to malabsorption in the enterocytes. Therefore, neonates suffering from cholestasis have limited uptake of the formulation. It has been indicated that the substitution of DSPE-PEG 2000 to the EPCs increases stability at a low pH. However, the PEGylated micelles still showed reduced uptake and transport of vitamin K by Caco-2 epithelial cells as compared to their non-PEGylated counterparts. In this work, peptides reverse-4F (R4F) and cRGD are conjugated to PEGylated micelles, respectively, using maleimide-thiol chemistry. R4F is an apolipoprotein (apo)A1 -mimetic peptide, the scaffolding protein of HDL. Since apoA1 is the main protein responsible for SR-B1 binding, it is hypothesized that through conjugation of R4F, cell binding and uptake is increased. Additionally, cRGD was conjugated to mixed micelles to compare its effects to R4F. cRGD is an integrin-binding motif that targets integrin αVβ3, expressed on M-cells. Conjugation of peptides to the micelles was determined using MALDI TOF MS, conjugation efficiency was determined by MicroBCA assay. Next, uptake studies of micelles with and without R4F and cRGD conjugates were performed on Caco-2 and U87-MG cell lines, respectively. The uptake of peptide conjugated micelles had no significant trend compared to uptake of micelles without peptide conjugate.

1. Introduction

1.1. Vitamin K$_1$

Vitamin K$_1$ (or phylloquinone) is a fat-soluble vitamin with a methylated naphthoquinone ring structure (Figure 1). Vitamin K$_1$ functions as a cofactor for the enzyme γ-glutamyl carboxylase, which catalyzes carboxylation of glutamic acid residues of various blood coagulation factors (II, VII, IX, X).[1] Adults obtain vitamin K through nutrition such as green leafy vegetables,[2,3] whereas fetuses and neonates receive vitamin K through the placental barrier and breastmilk, respectively. [1,4] Vitamin K deficiency is rare in healthy adults, however due to limited transport through the placental barrier prebirth and low concentrations in breastmilk, it is more often present in neonates.[1] This can lead to vitamin K deficiency bleeding (VKDB), further leading to intercranial bleeding, causing neurological damage or death.[5] VKDB mostly forms a risk at an early stage of life (0-6 months) and is predominantly present in infants that are exclusively fed with breastmilk.[3] Infants that are fed with milk formulas receive roughly 50 times more vitamin K (50 µg daily) than breastmilk receiving infants (0.1-2.1 µg daily).[4] This difference can also be observed in vitamin K plasma concentrations, with amounts of 0.13–0.24 mg/L for breastmilk fed infants and 4.4–6.0 mg/L for milk-formula fed children.[4] Infants with vitamin K deficiency can receive prophylactic vitamin K. Uptake of vitamin K is predominantly performed by enterocytes in the intestine and metabolism of vitamin K is facilitated by bile salts.[1] However, in case of cholestasis, pathophysiological conditions lead to limited amounts of bile release into the intestine, causing malabsorption of vitamin K.[6] Thus far, cholestasis is the only factor indicated to trigger VKDB.[3] Prophylactic vitamin K for infants with VKDB and cholestasis will be ineffective due to low bioavailability of the formulation.[1] Risk of VKDB is universal, but countries have different methods of handling its prevalence. In some countries, prevalence of VKDB in newborns is mandatory. In the United Stated, intramuscular injections are most common, whereas other European countries oral administration is preferred.[3]
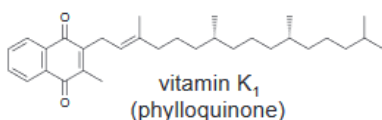

Figure 1. Chemical structure of vitamin K$_1$ (phylloquinone).[7]

1.2. Vitamin K Loaded Mixed Micelle Formulation

A commercial and clinically used vitamin K formulation is Konakion® mixed micelles Paediatric, prescribed for the prophylaxis and treatment of VKDB in neonates and infants through oral delivery.[1] Mixed micelles range in size from 5-60 nm, sufficient for transportation to the enterocytes and microvilli[1] The formulation contains egg phosphatidylcholine (EPC) or lecithin, glycocholic acid and vitamin K$_1$.[1] The EPC molecules consist of hydrophilic heads and hydrophobic tails, causing the phospholipids to self-assemble in bilayered structures in an aqueous environment. When bile salts are added (glycocholic acid), the structures form into mixed micelles. The inner micellar core is suited to insert the lipid soluble vitamin K. The mixed micelles are stabilized by the carboxylic acid group (pKa=3.8) of glycocholic acid at a pH above 4. The lower pH in the stomach leads to protonation of this group, resulting in large aggregates and coagulation.[1] These aggregates exit the stomach into the duodenum, an environment with a higher and therefore more suitable pH. Here, endogenous bile salts and pancreatic lipases solubilize the aggregates, leading to efficient absorption.[1] In case of cholestasis, reduced amounts of bile salts lead to ineffective solubilization and therefore, ineffective uptake. To improve the stability of the Konakion® mixed micelle formulation, the stability at a low pH should be increased while maintaining an effective uptake in the intestine.[1] The substitution of a polyethylene

glycol (PEG) lipid conjugate to mixed micelles was performed by van Nostrum *et al*, showing that adding DSPE-PEG 2000 to the EPCs (50/50 mol/mol percentage, respectively*)* increased stability at a low pH.[1] PEG 2000 is widely used in pharmaceutical drug delivery systems such as micelles, with PEG functioning as a steric barrier stabilizing the nanostructure at a neutral and low pH while showing biocompatibility, low toxicity and ease of excretion.[1,8] Although PEGylated micelles showed an increased stability, uptake pf the PEGylated micelles showed reduced uptake and transport of vitamin K by Caco-2 epithelial cells as compared to their non-PEGylated counterparts.[9]

1.3. Uptake Vitamin K

Mixed micelles loaded with vitamin K are taken up by at the brush-border membrane of enterocytes in the small intestine.[10] This energy dependent uptake is mediated by scavenger receptor class B type 1 (SR-B1) protein. SR-B1 is a multiligand membrane receptor and mediates transport of lipids, predominantly of cholesterol esters carried by high-density lipoprotein (HDL).[11,12] SR-B1 also facilitates lipid soluble vitamin uptake and is considered as a target for delivery of therapeutic agents, using reconstructed HDL (rHDL) particles for transportation to utilize the HDL-cholesterol ester uptake pathway.[13,14] SR-B1 is a homo-oligomeric glycoprotein [11] containing a hydrophobic channel through the cell membrane,[12] consisting of two intracellular and transmembrane domains, with most of the protein located extracellularly.[13] Dietary vitamin K is packed into micelles in the intestine, constructed with various lipids and bile salts.[3] On cell surfaces of enterocytes, SR-B1 can cluster into caveolae-like domains, where possibly intact micelles are endocytosed into the enterocytes.[9] After uptake, transportation to the basolateral site of the endothelium is carried out by cellular lipoproteins; chylomicrons (CMs) (Figure 2).[9] The CMs have an apoA and apoB-48 on the cell surface.[3] At the basolateral site, the CMs are secreted into lacteals and enter the lymphatic system.[3] The CMs will enter the blood circulation through the thoracic duct.[3]
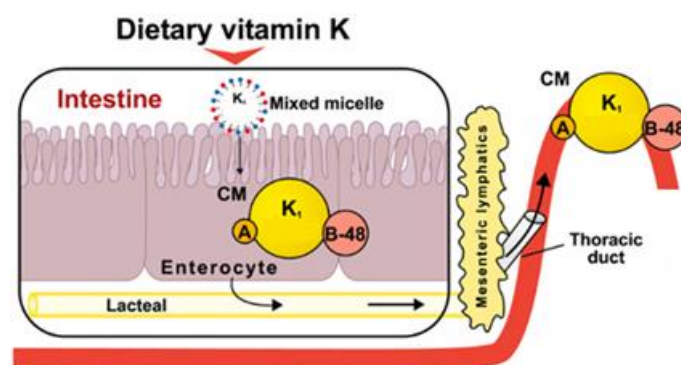

Figure 2. Illustration of dietary vitamin K uptake in the intestine. Vitamin K is packed into micelles and taken up by enterocytes, where the intracellular transport of vitamin K is conducted in CMs with apoA and apoB-48 on the cell surface. CMs are secreted into the lacteal and will enter the blood circulation through the thoracic duct. Adjusted from Shearer *et al*, Adv Nutr. 2012, 3(2):182–95.[3]

1.4. R4F Peptide Conjugation to Mixed Micelles

HDL mimicking nanoparticles (NPs) are important candidates for transportation of therapeutic agents due to its ability to transport hydrophobic molecules and anti-inflammatory benefits.[15,16] Reconstitution of HDL is of high interest for various therapies treating multiple diseases.[15] HDL has a diameter of 7-17 nm with a discoidal or spherical configuration depending on the presence of hydrophobic load in its hydrophobic core.[15] Reverse-4F (R4F) is an apoA1-mimetic peptide, the scaffolding protein of HDL.[15] ApoA1 is the main protein responsible for SR-B1 binding can function as a scaffold protein to bind cholesterols and free lipids and additionally functions as agonist for multiple anti-inflammatory pathways.[13,15] ApoA1-mimetic peptides contain 18-22 amino acids to form the apoA1 lipid binding domain, as a class-A amphipathic helical motif. Natural apoA1 can be obtained by purifying human plasma, a process that is very costly and difficult.[15] R4F is made combining the

bioavailability of apoA1-mimetic peptide 4F, while using different anti-inflammatory and lipid-binding properties.[15] R4F contains 18 amino acids (FAEKFKEAVKDYFAKFWD), while apoA1 contains over 240 amino acids, making synthesis and purification of R4F cost-effective compared to obtaining apoA1.[15] A study by Y. Kim *et al*.[15] compared NPs containing apoA1 and R4F, showing they were comparable in structure (Figure 3), composition and size, although apoA1 has various properties R4F does not mimic as effectively. Y. Kim *et al*. indicates that NPs containing R4F interact with SR-B1.[15] Other studies have shown that R4F has promising results in drug delivery and in *in vivo* studies using atherosclerotic mouse models.[15,17]
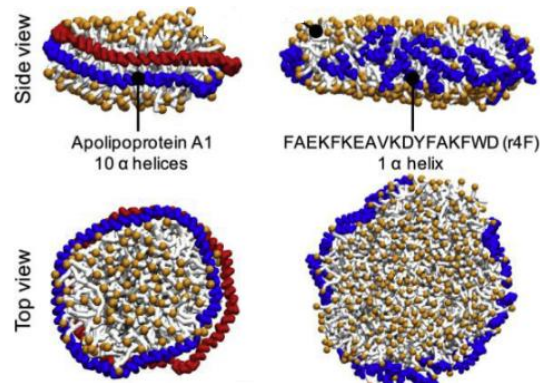

Figure 3. Peptide R4F (right) can self-assemble around lipids to form a discoidal NP similar to apoA1 (left). ApoA1 contains 10 α helices, R4F contains 1 α helix. Comparison made with molecular dynamics simulations. Adjusted from Sei *et al*, Biomaterials 2018 ,170:58–69.[15]

1.5. cRGD

Arg-Gly-Asp (RGD) is an integrin-binding motif that targets multiple integrins, predominantly αVβ3, which are transmembrane receptors consisting of two subunits that function as cell adhesion molecules.[19,20] Ligands bind to the extracellular domain of the integrins, whereafter the intracellular domain activates intracellular signaling pathways.[20] Naturally, RGD is present in extracellular matrix proteins such as fibrinogen.[21] The motif RGD is a dominant ligand to αvβ3, which is expressed on various cell types such as osteoclasts, smooth muscle cells, macrophages, activated endothelial cells and M cells.[23,24] Targeted drug delivery with micelles containing RGD motif have widely been used.[19] Research has shown that RGD-functionalized NPs targeting integrin αvβ3 increases delivery of anti-cancer agents. [25,26] Cyclic RGD (cRGD) has a higher affinity to αvβ3 and is therefore often used clinically than RGD.[19] Research has shown that PEGylated NPs with RGD conjugates had an increased interaction with M cells compared to non-targeted control groups.[24]

In this study, R4F and cRGD are conjugated to DSPE-PEG, respectively, using maleimide-thiol conjugation chemistry. This is performed by covalently binding cysteine to R4F or cRGD and maleimide to PEG, respectively.[18] Maleimide-thiol conjugation chemistry is frequently used due to the selectivity to thiol groups at a physiological pH, the high reactivity of maleimide and the stable thioether bond formation.[18] When R4F or cRGD is bound to the vitamin K loaded mixed micelle, it is hypothesized that cellular uptake will increase. PEGylated mixed micelles loaded with vitamin K were prepared, whereafter R4F or cRGD was conjugated to the micelles. Conjugation of peptides to the micelles was determined using MALDI TOF MS, subsequentially conjugation efficiency was determined by MicroBCA assay. Next, uptake studies of micelles with and without R4F and cRGD conjugates were performed using Caco-2 and U87-MG cell lines, respectively.

2. Materials and Methods

2.1. Materials

Glycocholic acid hydrate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid (EDTA), cysteine (Cys), ammonium bicarbonate (NH_4HCO_3), triethylamine, N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), 1-Hydroxybenzotriazole hydrate (HOBt), N,N-Diisopropylethylamine (DIPEA) and vitamin K_1 were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands). Lecithin (egg phosphatidylcholine, EPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-poly(ethylene glycol)2000 (DSPE-PEG 2000) were provided by LipoidGmbH (Ludwigshafen, Germany). DSPE-PEG2000 maleimide and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]-N-(Cyanine 5) (DSPE-PEG2000-Cy5)) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2H_2O$) and potassium phosphate monobasic (KH_2PO_4) were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands) to prepare 0.067 M phosphate buffer (0.35 g KH_2PO_4 and 1.45 g $Na_2HPO_4 \cdot 2H_2O$ in 100 ml RO-water, pH 7.3). Chloroform, methanol absolute, acetonitrile (ACN), dimethyl sulfoxide (DMSO), diethyl ether, and dichloromethane (DCM) were purchased from Biosolve (Valkenswaard, the Netherlands). Ethanol and sodium hydroxide (NaOH) were supplied by Merck KGaA (Darmstadt, Germany). Trifluoroacetic acid (TFA) and Phosphate Buffered Saline (PBS), 10x solution were obtained from ThemoFischer Scientific (Waltham, MA, USA). Cys-GGG-R4F was purchased from ChinaPeptides (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM, 4.5 g/mL glucose) and DMEM/F12 was purchased from GibCo BRL Life Technologies (Carlsbad, CA, USA). Syringe filters (0.2 μm) were obtained from Phenomenex (Torrance, CA). Vivaspin 2 (100, 300 kDa) were purchased from Satorius (Göttingen, Germany). Rhodamine-NHS, Hoechst 33342 and Micro BCA^{TM} Protein Assay Kit were purchased from Thermo Scientific (Illinois, USA). Dulbecco's phosphate buffered saline (8.0 g NaCl, 1.15 g Na_2HPO_4, 0.2 g KCl and 0.2 g KH_2PO_4 in 1 L of water, pH 7.4), non-essential amino acids (NEAA) and fetal bovine serum (FBS) were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands). Ultrapure water was prepared by a Synergy UV water delivery system from Millipore (Billerica, MA, USA).

2.2. Methods

2.2.1. Synthesis and Characterization of cRGD-FESS-Cys and Rhodamine-GG-R4F

Rhodamine-GG-R4F was synthesized based on standard solid peptide Fmoc solid-phase peptide synthesis on Fmoc-Asp(OtBu)-Wang Resin, 80-160 mesh. First, 0.2 g resin was pre-swelled by DCM and washed by dimethylformamide (DMF). To yield a loading of 0.75 mmol/g, 0.75 mmol amino acid dissolved in 5 ml DMF with 0.3g HBTU, 0.15g HOBt and 270μl N,N-Diisopropylethylamine (DIPEA) were added and reacted for 1 h at room temperature. Fmoc-groups were removed before each coupling and after final coupling by treatment with 5 ml 20% piperidine in DMF for 10 min. The peptide resin was washed 3 times with 5ml DMF and DCM respectively after synthesis, dried at room temperature and stored at -20 °C. Next, 0.025 mmol peptide resin was incubated with rhodamine-NHS (25 mg) and 50 μl triethylamine in 2 ml DMF for 4 h, then washed 3 times by DMF and DCM for 3 times, respectively. Next, the peptide was cleaved in 3 ml TFA/H_2O/TIPS/1,2-ethanedithiol (92.5/2.5/2.5/2.5, *v/v*) for 3 h. Diethyl ether(50ml) was added to precipitate the peptide, after which it was centrifuged at 6000 x *g* for 5 min and washed 3 times by 50ml diethyl ether. The peptide was dissolved in 30 ml water, after which 3 ml 1M NH_4HCO_3 was added to obtain a clear solution. Subsequently water was added to refill to 100 ml, then the peptide was freeze dried. Rhodamine-GG-R4F was purified using ReproSil-Pur C18 column (10 μm, 250 × 22 mm) eluted with water-ACN gradient 5-100% (10 mM NH_3HCO_3) for 40 min at flow rate of 15.0 ml/min with ultraviolet (UV) detection at 220 nm and 280 nm. cRGD-phenylalanine-

glutamic acid-serine-serine-cysteine (cRGD-FESS-Cys) was prepared using the same method. The purity was characterized by Ultra Performance Liquid Chromatography (UPLC) and molecular weight was confirmed using electrospray ionization-liquid chromatography (ESI-LC)/mass spectrometry (MS) instrument (Bruker Q-TOF mass spectrometer, Bremen, Germany)) and Matrix-Assisted Laser Desorption Ionization–Time of Flight (MALDI-TOF) MS (UltrafleXtreme, Bruker, CA, US). Purity of rhodamine-GG-R4F and Cys-GGG-R4F was confirmed by analytical UPLC using Waters BEH C18 column (1.7 μm, 2.1 × 50 mm) eluted with gradient 20–80% ACN (0.1% TFA) in 10 min, followed by 20% ACN (0.1% TFA) for 5min, at a flow rate of 0.25 ml/min and UV detection at 220 and 280 nm. Purity of cRGD-FESS-Cys was confirmed by analytical UPLC using Waters BEH C18 column (1.7 μm, 2.1 × 50 mm) eluted with a gradient 5-60% ACN (0.1% TFA) in 10 min, followed by 5% ACN (0.1% TFA) for 5min, at a flow rate of 0.25 ml/min and UV detection at 220 and 280 nm.

2.2.2. Preparation of Mixed Micelles

To prepare 50% PEGylated mixed micelles with and without maleimide, various amounts of lipids (DSPE-PEG 2000, DSPE-PEG2000-maleimide, DSPE-PEG-Cy5 and EPC) and glycocholic acid hydrate were dissolved in 0.6 ml methanol and 1.4 ml chloroform in a round-bottom flask. Vitamin K_1 (378.3 mg) was dissolved in 7.57 ml chloroform, of which 25 μl was added to the lipid mixture. Using a rotary evaporator, the solvent was evaporated under reduced pressure at 40°C for 20 min to create a film. Next, the film was put under nitrogen-blow for 10 min and hydrated by adding 0.5 ml 0.067 M phosphate buffer to a 25 mM total lipid concentration (Table 1). After 4 h magnetically stirring at room temperature, a transparent dispersion was obtained, that was extruded through a PTFE syringe filter of 0.2 μm for 3 times. Two formulations were prepared, the first containing a lipid molar ratio of 50/50 of DSPE-PEG/EPC (50%PEG), the second containing a lipid molar ratio of 35/15/50 of DSPE-PEG/DSPE-PEG-maleimide/EPC (35%PEG-15%mal) (Table 1). During the entire procedure, the dispersions were covered with aluminized foil to prevent degradation of vitamin K and Cy5 red fluorescence.

Table 1. Components of vitamin K loaded 50% PEGylated mixed micelles with and without maleimide.

Concentration	Formulations	
	50%PEG	35%PEG-15%mal
EPC (mM)	12.50	12.50
DSPE-PEG 2000 (mM)	12.50	8.75
DSPE-PEG-maleimide (mM)	0	3.75
DSPE-PEG-Cy5 (mM)	0.025	0.025
Glycocholic acid hydrate (mM)	33.00	33.00
Vitamin K (mM)	5.55	5.55
DSPE-PEG/DSPE-PEG-mal/EPC (*mol/mol/mol*)	50/0/50	35/15/50

2.2.3. Conjugating Peptides to Mixed Micellar Formulations

Peptides R4F and cRGD were conjugated to the formulations, respectively, whereafter cysteine was conjugated to the formulations to block unreacted maleimide groups, finally formulations were washed using vivaspin (Figure 4). In total, seven different formulations were obtained (Table 2). R4F was dissolved in 0.5 mM acetic acid/30 mM HEPES, 1.2 mM EDTA (2/1, *v/v*, pH 7.0) and cRGD was dissolved in 10mM NaOH/H_2O/10xPBS (1/8/1, *v/v*, pH 7.4) with concentrations of 250 μg/ml. Next, 100 μl 35%PEG-15%mal was added to the R4F or cRGD dispersion, to thiol/maleimide (1.2/1.0, *mol/mol*). Additionally, 100 μl of 50%PEG was incubated with equal amounts of peptides as a control, to investigate if peptides can conjugate to the formulation in absence of maleimide. The formulations

were put under nitrogen blow for 5 min to avoid disulfide bond formation, and magnetically stirred for 1 h at room temperature, subsequentially at 4°C overnight. Next, a cysteine buffer was made by dissolving cysteine in 0.067 M phosphate buffer to a concentration of 10 mg/ml. The peptide conjugated mixed micelles were incubated with cysteine buffer to thiol/maleimide (3/1, *mol*/*mol*) for 1 h at room temperature while magnetically stirring. Additionally, the 35%PEG-15%mal mixed micelle dispersion was incubated with the cysteine buffer to an equal ratio thiol/maleimide using the same conditions, to obtain 35%PEG-15%mal-cys, a non-targeted control.

Table 2. Final types of formulations with and without peptide and cysteine conjugation.

Mixed Micelle	R4F	cRGD	Cysteine
50%PEG	*No*	*No*	*No*
35%PEG-15%mal	*No*	*No*	*No*
50%PEG-R4F	*Yes*	*No*	*Yes*
50%PEG-cRGD	*No*	*Yes*	*Yes*
35%PEG-15%mal-cys	*No*	*No*	*Yes*
35%PEG-15%mal-R4F	*Yes*	*No*	*Yes*
35%PEG-15%mal-cRGD	No	Yes	Yes

The formulations were washed in 5 rounds with 300 µl 0.067 M phosphate buffer, respectively, using vivaspin 2 (100kDa) at 4000 x *g* for 20 min. In order to remove possible aggregations, formulations incubated with R4F were washed with vivaspin for an extra round using vivaspin 2 (300kDa). The washing buffers were collected in Eppendorfs and kept at -20℃ until further use. The formulations were resuspended in 0.067 M phosphate buffer.
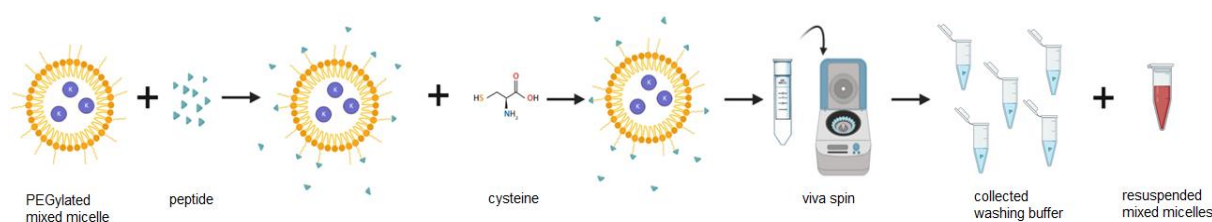

Figure 4. Illustration of peptide conjugation to vitamin K loaded PEGylated mixed micelles.

2.2.4. Characterization of Mixed Micelles

To determine the size of the micelles, 25 µl of formulations 50%PEG, 35%PEG-15%mal, 35%PEG-15%mal-cys, 35%PEG-15%mal-R4F and 35%PEG-15%mal-cRGD was diluted in 1 ml 10 mM HEPES buffer (pH=7.4), respectively, and subsequentially measured by Dynamic Light Scattering (DLS; Zetasizer 4000, Malvern Instruments, Malvern, UK*)* at 25 °C with an angle of 90°. Next, the zeta potential of the same dispersions was measured by Zetasizer (Malvern Instruments Ltd.) at 25 °C, using a standard (DTS1235,−42.0 ± 4.2 mV, Malvern Instruments, UK) for calibration of the instrument. Determination of DSPE-PEG-Cy5 concentrations in the same formulations as above was performed using fluorescence spectrophotometer (Spectrofluorometer FP-8300, Jasco Corporation, Tokyo, Japan), to finally calculate the recovery of the micelles after vivaspin centrifuge. Of the formulations, 5 µl was diluted 10 times by addition of 45 µl ethanol and 45 µl DMSO, after which they were vortexed for 5 min and centrifuged 10 min at 8000 x *g*. A calibration curve was prepared by diluting DSPE-PEG-Cy5 in ethanol and DMSO (1:1) with concentrations 10 to 0.2 ug/ml. Subsequently, 60 µl of the supernatants were transferred into a 384 well and measured at an excitation wavelength of 640 nm and an emission wavelength of 670 nm. The recovery was calculated as:

$$Recovery\ (\%) = \left(\frac{[DSPE - PEG - Cy5\ in\ the\ superntant]}{[DSPE - PEG - Cy5\ added\ to\ formulation]} \right) x100\%$$

2.2.5. MALDI TOF MS to determine peptide conjugation to DSPE-PEG-maleimide

MALDI-TOF MS was used to determine if peptides R4F or cRGD were successfully conjugated to DSPE-PEG-maleimide. Five microliters of formulations 35%PEG-15%mal-R4F and 35%PEG-15%mal-cRGD and 50%PEG as a control were respectively diluted in 5 µl chloroform and 5 µl DMSO and vortexed for 5 min. Next, 1 µl of the dispersion was mixed with 1 µl CMBT (20 mg/ml, which was diluted in chloroform/methanol/H_2O (4/4/1, *v/v/v*)) and dropped to MALDI target plate MTP 384 ground steel, and measured by MALDI TOF MS. The results were analyzed by Bruker data analysis software.

2.2.6. Determination of Peptide Conjugation Efficiency

To determine the peptide concentration in the washing buffers used for viva spin, formulations 35%PEG-15%mal-cRGD and 35%PEG-15%mal-R4F were prepared according to 2.2.3., but incubation with cysteine was left out. Next, Micro BCA assay was performed according to protocol of the manufacturer. Working reagents Micro BCA™ Reagent A, -B and -C (25/24/1, *v/v/v*) were mixed. Next, 150 µl of the collected washing buffers of vivaspin was loaded on a 96 well plate and 150 µl working reagent was added. The plate was shaken for 30 seconds and incubated for 2 h at 37°C. A standard curve was made with concentrations ranging of 3.9-125 µg/ml. Spectrophotometer (SPECTROstar Nano, BMG Labtech, Ortenberg, Germany) was used and the absorbance was measured at 562 nm. Data was analyzed with SPECTROstar Nano. The conjugation efficiency was calculated as:

$$Conjugation\ efficiency(\%) = \left(1 - \left(\frac{[ligand\ in\ the\ supernatant]}{[Ligand\ added\ for\ the\ conjugation\ reaction]}\right)\right) x 100\%\ ^{[22]}$$

R4F and cRGD were respectively added in excess to the micelles compared to the maleimide groups, with a molar ratio of maleimide/peptide as 1/1.2. Therefore, the maximum conjugation efficiency was 83.3%.

2.2.7. Cell Culture

Caco-2 cells were cultured in DMEM (4.5 g/ml glucose) supplemented with 10% fetal bovine serum (FBS) and 1% non-essential amino acid (NEAA) in an incubator at 37°C with 5% CO_2. HeLa cells and U87-MG cells were cultured with DMEM (4.5 g/ml glucose) and DMEM/F12 supplemented with 10% FBS respectively, under the same conditions.

2.2.8. Uptake of Rhodamine-GG-R4F in HeLa cells with Overexpression of SR-B1

HeLa cells were seeded in a 96-well plate with a density of 1×10^4 cells per well and incubated overnight at 37 °C. The cells were transfected with a plasmid containing SR-B1 cDNA with a C-terminal green fluorescence protein (GFP) tag using Lipofectamine 3000 according to the protocol provided by the manufacturer. Briefly, 20 µl of the lipofectamine/cDNA dispersion containing cDNA (0.1 µg), 0.9 µl lipofectamine 3000 and 0.2 µl P3000 reagent were added to each well and incubated for 48 h at 37 °C to obtain transfected Hela cells. The transfected HeLa cells were incubated with 50 µl rhodamine-GG-R4F (10.0, 5.0 and 2.5 µg/ml, respectively) for 2 h at 37°C in blank DMEM, respectively. To stain cell nuclei of the HeLa cells, 10 µl Hoechst 33342 (1:100 diluted in DMEM) was added to each well and incubated with the cells for 15 min at 37 °C. Next, the medium was removed and the cells were washed 3 times with PBS. Subsequently, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature, then washed with PBS for 3 times. Images were acquired using a confocal microscope (Yokogawa CV 7000s, Yokogawa Electric Corporation, Tokyo, Japan) with 60× objective. The excitation wavelengths were 405, 488 and 561 nm, while the corresponding acquisition channels were 445 ± 45, 525 ± 50 and 600 ± 37 nm respectively. Columbus was used to analyze image.

2.2.9. Cell Uptake Studies of R4F- and cRGD Conjugated Mixed Micelles

Caco-2 cells were seeded in a 96-well plate with a density of 1 x 10⁴ cells per well and incubated overnight at 37 °C. A 96-well plate with U87-MG cells was prepared similarly. Formulations 50%PEG, 35%PEG-15%mal-cys, 35%PEG-15%mal-cRGD and 35%PEG-15%mal-R4F, all containing a fluorescently labeled peptide (DPSE-PEG-Cy5), were diluted in blank DMEM to a concentration of 1 mM total lipid. Caco-2 cells were incubated with 50 µl of formulations 50%PEG, 35%PEG-15%mal-cys and 35%PEG-15%mal-R4F in DMEM for 30 min at 37°C. U87-MG cells were incubated with 50 µl of formulations 50%PEG, 35%PEG-15%mal-cys and 35%PEG-15%mal-cRGD in DMEM for 2 h at 37 °C. To stain cell nuclei, 10 µl Hoechst 33342 (1:100 diluted in DMEM) was added to each well and incubated with the cells for 15 min at 37 °C. The following steps were identical to the steps after Hoechst 33342 staining in 2.2.8.. The excitation wavelengths were 405, and 640 nm, while the corresponding acquisition channels were 445 ± 45, and 670 ± 37 nm respectively. Columbus was used to analyze image.

3. Results and Discussion

3.1. Characterization of cRGD-FESS-Cys and Rhodamine-GG-R4F

The theoretical mass values for rhodamine-GG-R4F, cys-GGG-R4F and cRGD-FESS-Cys are 2796, 2543, 881 g/mol respectively. Figure 5 showed Rhodamine-GG-R4F m/z peak of 1396.6, equal to (M+2H)²⁺/2. In Figure 6, Peaks were detected at 2565 (cys-GGG-R4F, left) and 879.4 (cRGD-FESS-Cys, right), corresponding to the (M+Na)⁺and (M+H)⁺, respectively. Purity of peptides was determined by UPLC, as shown in Figure7. Rhodamine-GG-R4F (retention time: 6.5 min) and cys-GGG-R4F (retention time: 6min) both showed good purity (>90%) with one dominant peak. However, cRGD-GG-Cys (retention time: 1.8 min) showed poor purity(< 50%), which requires further optimization.
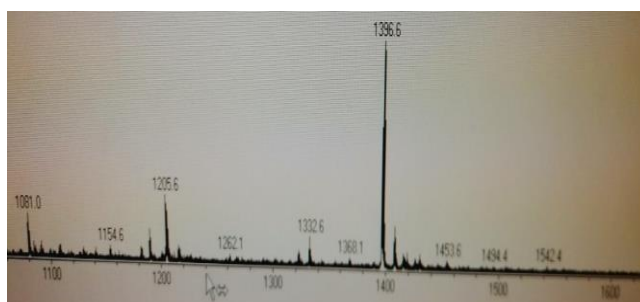

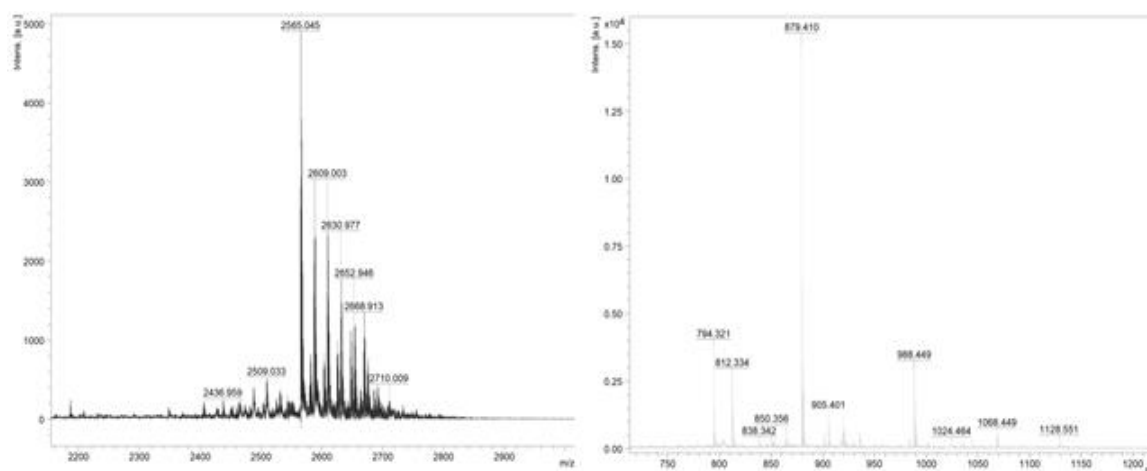**Figure 5.** MS analysis of rhodamine-GG-R4F by ESI-LC/MS instrument.

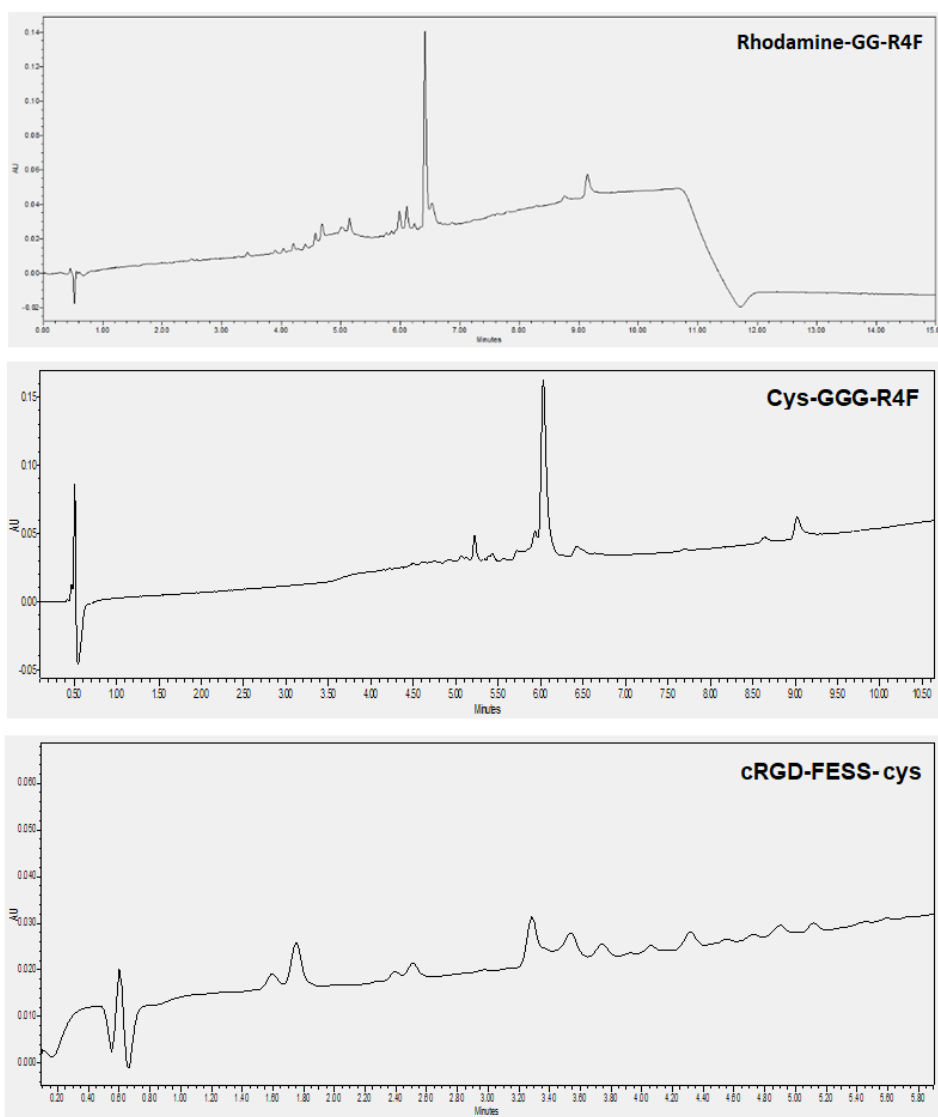**Figure 6.** MS analysis of Cys-GGG-R4F (left) and cRGD-FESS-Cys (right) measured by MALDI TOF MS.

Figure 7. Analytical UPLC results of rhodamine-GG-R4F, Cys-GGG-R4F and cRGD-FESS-Cys at UV detection 220 nm.

3.2. Characterization of Mixed Micelles

Vitamin K loaded mixed micelles were prepared and thereafter conjugated to a peptide, as described in *2.2.2.* and *2.2.3.,* obtaining seven different formulations (Table 2). Characterization was performed by monitoring the size and zeta potential of formulations with different compositions, before and after incubation with R4F, cRGD and cysteine. Formulations 50%PEG, 35%PEG-15%mal, 35%PEG-15%mal-cys, 35%PEG-15%mal-R4F and 35%PEG-15%mal-cRGD were diluted in 10 mM HEPES (pH=7.4) and measured by DLS and Zetasizer (Table 3). Conjugation of R4F or cRGD to the mixed micelles did not significantly alter their size and charge. The average size of the micellar dispersions was between 14.9 and 24.8 nm (Table 3). The average size of a vitamin K loaded mixed micelle with a EPC/DSPE-PEG molar ratio of 50/50 in absence of a conjugated peptide should be close to 11.0 ± 1.4 nm and the average PDI should be around 0.26. [1] The increase in size and polydispersity (PDI) indicate aggregation of the micelles, caused by aggregated R4F on the surface of the micelles. The formulations had a negative zeta potential with values between -7 and -10 mV. Similar zeta potential values of RGD derivatives conjugated to NPs have been reported by others .[18, 22, 27]

Table 3. Size, zeta potential and recovery of vitamin K loaded mixed micelles with different compositions, with or without conjugation of R4F, cRGD and cysteine[a].

Sample	Size (d.nm)	PDI	Zeta potential (mV)	Recovery[b] (%)
50%PEG	14.9 ± 1.10	0.28 ± 0.04	-7.1 ± 0.5	100.0
35%PEG-15%mal	16.0 ± 0.35	0.29 ± 0.01	-8.1 ± 0.2	NA[c]
35%PEG-15%mal-cys	22.9 ± 1.60	0.32 ± 0.05	-10.0 ± 1.0	38
35%PEG-15%mal-R4F	18.0 ± 0.18	0.36 ± 0.01	-9.0 ± 1.0	32
35%PEG-15%mal-cRGD	24.8 ± 2.30	0.36 ± 0.01	-10.3 ± 0.1	41

n=1. [a] Conjugation of R4F and cRGD was performed in molar ratio of 1/1.2 maleimide/thiol and cysteine in 1/3 maleimide/thiol. [b] Recovery (%)=([DSPE-PEG-Cy5 in the superntant]/[DSPE-PEG-Cy5 added to formulation])x100%. [c] Not applicable. All data are mean ± SD.

By determining DSPE-PEG-Cy5 concentrations, recovery of the micelles after vivaspin, as described in *2.2.3.*, could be calculated. A calibration curve was prepared by diluting DSPE-PEG-Cy5 in ethanol and DMSO(1:1, v:v) with concentrations 10 to 0.2 µg/ml (Figure 8). Recovery of the formulations washed by vivaspin was low (32-41%) compared to the control that was not washed by vivaspin (100%), shown in Table 3. Low recovery is determined because low quantities of peptide were available, leading to small volumes of micelles that could be incubated with the peptides. During the transfer of the dispersions, relative large amounts of liquid were lost.
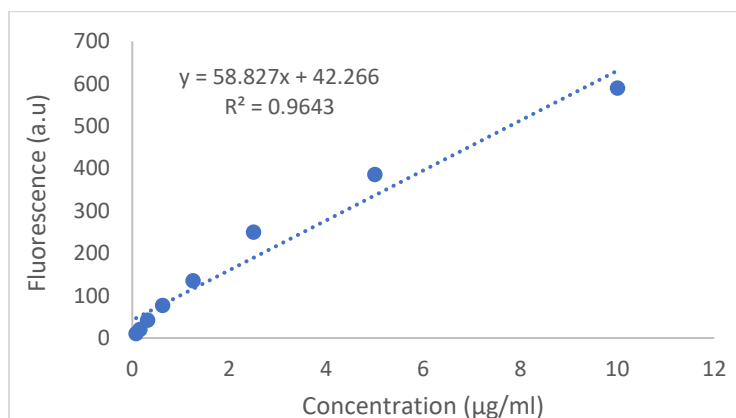

y = 58.827x + 42.266
R² = 0.9643

Figure 8. Calibration curve DSPE-PEG-Cy5 (10 to 0.2 µg/ml) in ethanol and DMSO (1:1, v:v), measured at excitation wavelength of 640 nm and emission wavelength of 670 nm.

3.3. MALDI TOF MS to determine peptide conjugation to DSPE-PEG-maleimide

MALDI TOF MS was used to confirm formation of DSPE-PEG-R4F and DSPE-PEG-cRGD by maleimide-thiol chemistry. The theoretical molecular weight of DSPE-PEG-R4F and DSPE-PEG-cRGD are 5458 and 3813 g/mol, respectively. The distribution of DSPE-PEG peaks was clearly seen in Figure 9 and Figure 10, with 44 (one PEG unit) difference between each neighboring peak. For 35%PEG-15%mal-R4F (Figure 9) and 35%PEG-15%mal-cRGD (Figure 10), significant peaks around calculated molecular weight appeared. This indicates that the peptides are successfully conjugated to DSPE-PEG-maleimide.
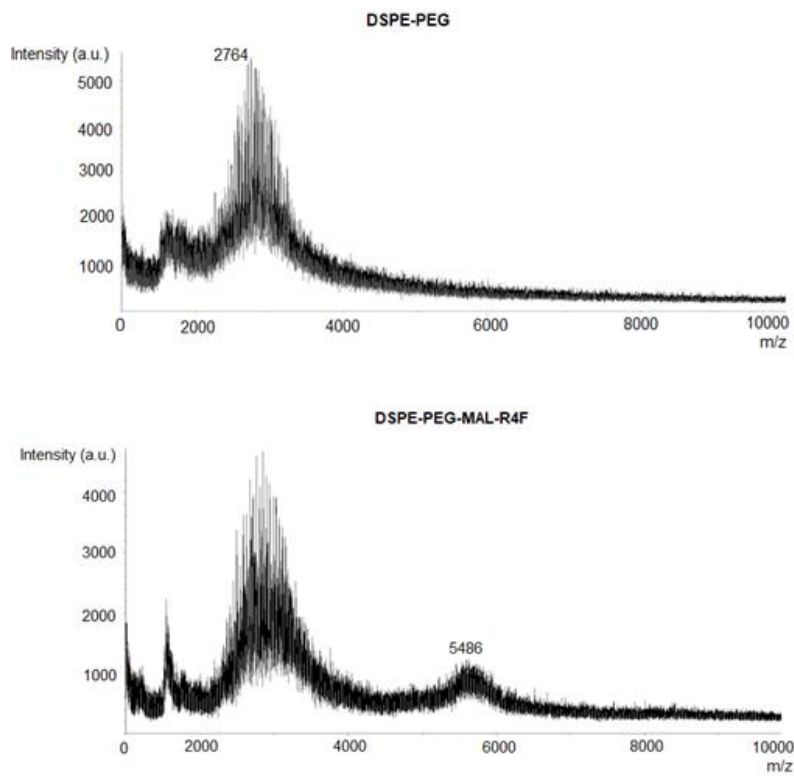
Figure 9. MALDI TOF MS results of 50%PEG and and 35%PEG-15%mal-R4F measured at 2000-20000 range.
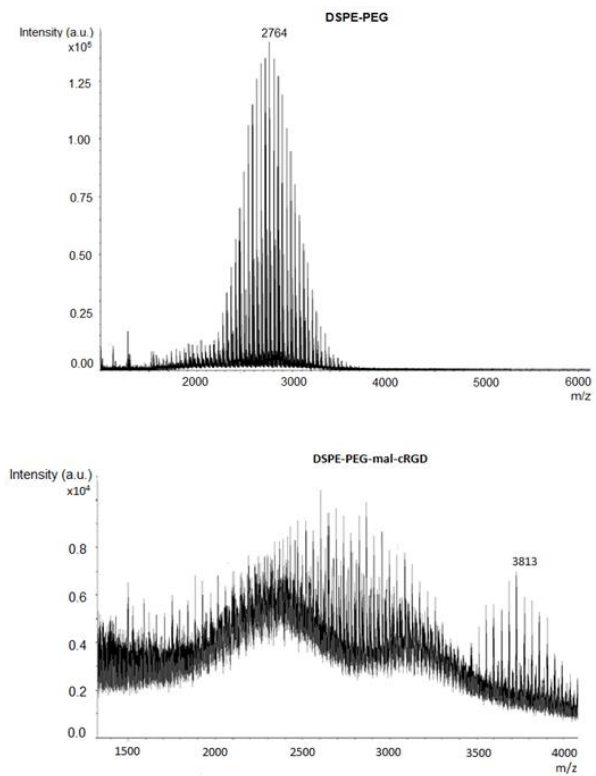

Figure 10. MALDI TOF MS results of 50%PEG and 35%PEG-15%mal-cRGD measured at 1000-8000 range.

3.4. Determination of Peptide Conjugation Efficiency

MicroBCA assay was performed to determine peptide concentrations of R4F and cRGD, respectively, in the washing buffers used for vivaspin. The conjugation efficiency of R4F and cRGD to the mixed micelles was calculated using the equation stated in *2.2.6.*. The micelles were incubated with R4F or cRGD, but not with cysteine. R4F and cRGD were respectively added in excess to the micelles compared to the maleimide groups, with a molar ratio of maleimide/peptide as 1/1.2. Therefore, the maximum conjugation efficiency was 83.3%. The conjugation efficiency of both peptides were normalized to this value. A standard curve was made with R4F with concentrations ranging of 3.9-62.5 µg/ml, subsequentially with cRGD with concentrations ranging of 3.9-125 µg/ml (Figure 10).
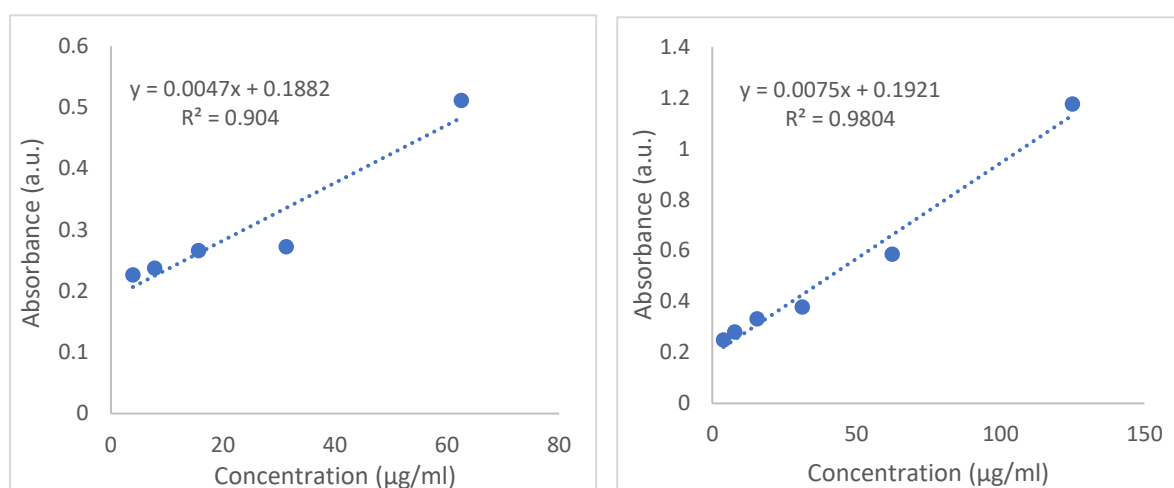

Figure 11. Calibration curve R4F (3.9 to 62.5 µg/ml) (left) and calibration curve cRGD (3.9-125 µg/ml*)* (right). Absorbance was measured at 562 nm.

The concentrations of washed out peptides was determined per round of vivaspin and added up per formulation, to calculate the total concentration of washed out peptide (Table 4). For micelles incubated with R4F, an extra round of vivaspin (300kDa) was performed to wash out aggregates. With a maximum conjugation efficiency of 83.3%, it was expected that 16.7% of the peptide was washed out. 50%PEG that was incubated with peptides was used as a control to determine of 100% peptide is washed out and conjugation efficiency is 0%, due to the absence of maleimide. Conjugation efficiency of R4F was 72% for 35%PEG-15%mal, but also for the control group 50%PEG, meaning that free R4F can be stuck in the micelle. The control group 50%PEG that was incubated with cRGD showed that 100% of the peptide was washed out, indicating 0% conjugation efficiency. For 35%PEG-15%mal, conjugation efficiency of cRGD was 50%.

Table 4. Conjugation efficiency of cRGD and R4F to the micelles.

Formulation	Washed out peptide (%)		Conjugation Efficiency (%)[a]
	100 kDa	300 kDa	
50%PEG-R4F	14	4	72
35%PEG-15%mal-R4F	18	0	72
50%PEG-cRGD	100	NA [b]	0
35%PEG-15%mal-cRGD	50	NA [b]	50

n=1. [a]Conjugation efficiency = (1–([ligand in the supernatant]/[ligand added in the conjugation reaction])) ×100%). [b] Not applicable.

3.5. Cell Uptake of Rhodamine-GG-R4F in HeLa cells with Overexpression of SR-B1

To investigate the affinity of free R4F peptide with SR-B1, HeLa cells were transfected with a plasmid containing SR-B1 cDNA. The HeLa cell culture is a widely used model that hardly expresses SR-B1, however it has been shown to successfully express SR-B1 after incubation with a plasmid containing SR-B1 cDNA[28 29]. Since HeLa cells have very low expression of SR-B1 before transfection, almost all SR-B1 showed green fluorescence after HeLa cells were transfected. Transfected HeLa cells were incubated with 2.5 and 5.0 µg/ml Rhodamine-GG-R4F in blank DMEM for 2h at 37°C (Figure 12). HeLa cells with GFP signal showed slight higher uptake than cells without GFP. This trend is more significant when cells incubated with 10 µg/ml rhodamine-GG-R4F (Figure 12). It is clearly seen that a significant amount of red fluorescence is located at cells overexpressing SR-B1 compared to cells that have no overexpression of SR-B1. This indicates that free R4F successfully binds to SR-B1.
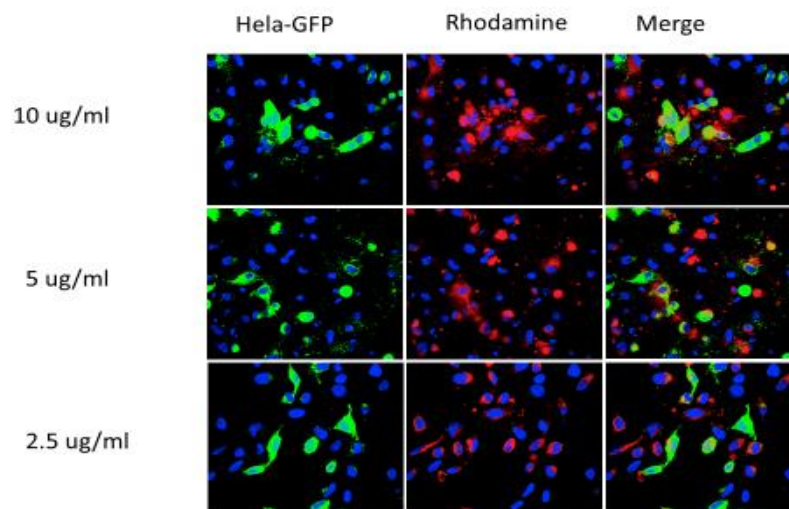

Figure 12. Representative confocal microscopic images of HeLa cells overexpressing SR-B1 upon incubation at 37 °C with Rhodamine-GG-R4F (2.5 ,5.0 and 10 µg/ml). SR-B1 is stained by GFP (green), R4F by rhodamine (red), cell nuclei by Hoechst 33342 (blue).

3.6. Cell Uptake Studies of R4F- and cRGD Conjugated Mixed Micelles

Caco-2 cells are human adenocarcinoma cells that express SR-B1[30], while U87-MG cells are human glioblastoma astrocytoma cells that express integrin αVβ3[31]. These two cell lines are selected to investigate the function of R4F and cRGD conjugated on the surface of PEGylated mixed micelles. HeLa cell line was selected in *2.2.8.* for uptake studies of free R4F, since it only significantly expresses SR-B1 after transfection with a plasmid contraing SR-B1 cDNA, but also cDNA included a GFP tag. By this way, it was possible to image the expression of SR-B1. The Caco-2 cell line is sufficient for this study since it expresses SR-B1 without transfection and it is not necesarry visualize the location of SR-B1 using GFP. Caco-2 cells were incubated for 30 min at 37°C with 50%PEG, 35%PEG-15%mal-cys and 35%PEG-15%mal-R4F labeled by Cy5 respectively. U87-MG cells were incubated for 2h at 37°C with 50%PEG, 35%PEG-15%mal-cys and 35%PEG-15%mal-cRGD labeled by Cy5 respectively. As shown in Figure 12, conjugation with cysteine showed no influence on uptake in both Caco-2 cells and U87-MG cells, compared with 50%PEGylated micelles. However, a decreasing trend in uptake was detected using R4F conjugated formualtions compared to the non-targeted control. Conjugation of R4F possibly reduces the affinity of 50%PEGylated mixed micelles with SR-B1. On the other hand, considering that free R4F was not removed completely from the micelles, it could induce competition with PEGylated mixed micelles interacting with SR-B1, resulting in inhibited uptake. Micelles conjugated to cRGD did also not show a significant trend in the uptake compared to the non-targeted contol, although a small amount of cells showed stronger association of red fluorescence. This may be caused by aggregations of

micelles. The low purity of cRGD used in this study may influence the affinity. Last, for small micelles with a size of less than 25 nm, it is more difficult to promote active targeting than bigger size NPs such as liposomes[32]. This requires further exploring binding and uptake studies using micelles with different ligand densities.
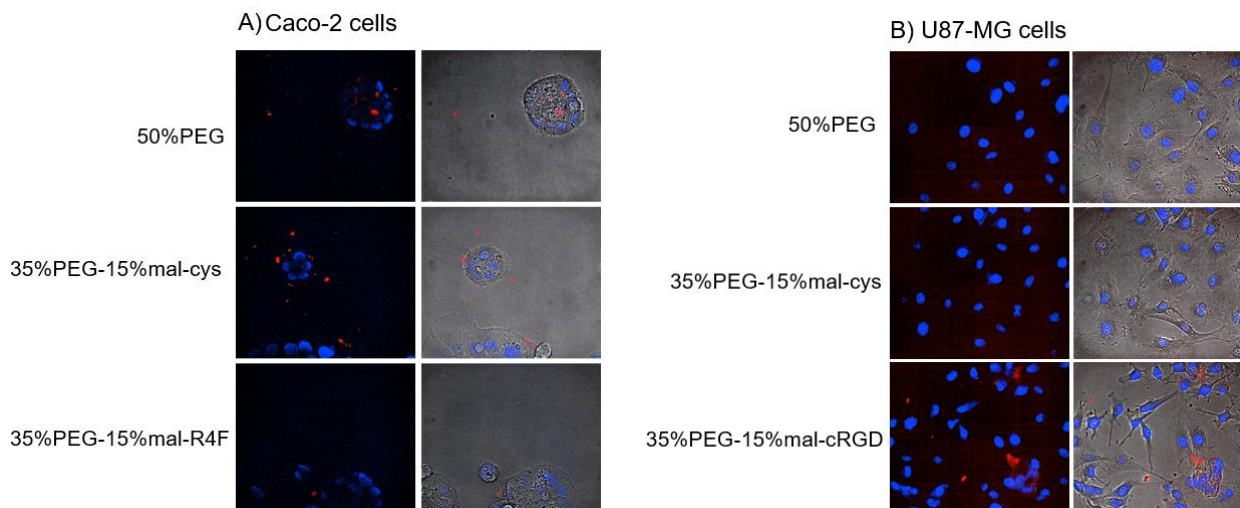

Figure 13. (A) Uptake studies of Caco-2 cells incubated with formulations 50%PEG, 35%PEG-15%mal-cys and 35%PEG-15%mal-R4F, for 30 min at 37°C. (B) Uptake studies of U87-MG cells incubated with formulations 50%PEG, 35%PEG-15%mal-cys, 35%PEG-15%mal-cRGD, for 2 h at 37°C. Micelles are stained by Cy5 (red), cell nuclei by Hoechst 33342 (blue), and bright-field images are merged in the right columns.

4. Conclusions

The results presented in this work show that free R4F has an increased binding to SR-B1 overexpressing cells compared to cells have no overexpression of SR-B1. Although we have indicated that peptides R4F and cRGD can be conjugated to DSPE-PEG-maleimide using maleimide-thiol click reaction, the conjugation efficiency of R4F was not optimal. This is possibly due to free R4F that gets into the micelle. The conjugation efficiency of cRGD was better, and can possibly be optimized using a higher purity of cRGD. Uptake studies to determine if R4F or cRGD conjugated mixed micelles have an increased uptake in Caco-2 cells or U87-MG cells, respectively, have not shown any significant difference compared to the non-targeted control groups. For R4F conjugated micelles, this is possibly due to aggregates of R4F that arise when R4F is dissolved in buffer, which are difficult to separate from the micelles. In the future, DSPE-PEG-R4F conjugates could be prepared, so that this component can be used directly during the preparation of mixed micelles. In this way, dissolving and conjugating steps can be removed, preventing the utilization of free R4F to influence the results. For future research of cRGD conjugated micelles, synthesis of pure cRGD is necessary. When these steps have been taken, further research can be conducted for binding and uptake of micelles with different ligand densities. Successfully conjugating ligands to the micelles can be very promising in improving the uptake of vitamin K loaded mixed micelles, but also for other PEGylated nanocarriers transporting lipophilic therapeutic agents.

Acknowledgement

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