POLITECNICO DI TORINO

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Formulazione di Nanoemulsioni Stabilizzate da Nanocristalli di Cellulosa

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Sommario

S1. Introduzione

I nanocristalli di cellulosa (CNCs) sono una nuova classe di nanoparticelle disponibili in commercio, comunemente derivate dal legno. Grazie alla loro origine naturale e alle loro proprietà funzionali, sono di grande interesse nell'ambito di applicazioni commerciali come i prodotti cosmetici e per la cura della persona. Negli ultimi anni, diversi studi hanno segnalato la capacità dei CNCs di stabilizzare le emulsioni grazie alla loro bagnabilità intermedia e alle dimensioni nanometriche, che gli consentono di adsorbirsi all'interfaccia olio-acqua permettendo la stabilità dell'emulsione grazie al cosiddetto meccanismo di Pickering. Le emulsioni di Pickering sono di particolare interesse accademico e industriale. Ciò è dovuto al fatto che, rispetto alle emulsioni stabilizzate dai comuni emulsionanti a basso peso molecolare, le emulsioni di Pickering sono note per mostrare una maggiore stabilità verso la coalescenza delle gocce dell'emulsione e l'instabilità da *Ostwald ripening*. Inoltre, l'esistenza di uno strato denso di particelle in corrispondenza dell'interfaccia delle gocce ha un impatto sulla capacità di incapsulamento delle gocce dell'emulsione, aprendo la possibilità di utilizzare questi sistemi in applicazioni ad uso topico per il rilascio controllato di un principio attivo.

I CNCs, da soli, hanno dimostrato di avere la capacità di stabilizzare emulsioni di olio in acqua (o/w), mentre i CNCs, la cui bagnabilità è stata regolata dall'adsorbimento di una specie tensioattiva, hanno dimostrato di avere la capacità di stabilizzare sia le emulsioni o/w che le w/o (a seconda della bagnabilità). In generale, i risultati disponibili in letteratura indicano che, rispetto alle emulsioni stabilizzate esclusivamente da CNCs, una buona stabilità dell'emulsione può essere raggiunta a concentrazioni complessive di CNCs più basse nelle emulsioni prodotte da CNCs la cui bagnabilità è stata modificata dall'adsorbimento di specie tensioattive. Indipendentemente dalla natura della fase continua e dispersa (emulsioni o/w o w/o), la maggior parte degli studi che trattano questi sistemi presentano dimensioni delle gocce nell'intervallo dei micron. Solo di recente è stata dimostrata la possibilità di realizzare emulsioni stabilizzate da CNCs con gocce di diametro inferiore al micron. Ciò è stato ottenuto attraverso l'uso della microfluidizzazione, una tecnica di emulsificazione ad alta pressione ben consolidata (Vedi sezione 3.3.2.2.).

Nel contesto delle applicazioni nutraceutiche, cosmetiche e farmaceutiche, emulsioni con dimensioni medie delle gocce nell'intervallo dei nanometri mostrano una serie di vantaggi rispetto alle loro controparti con dimensioni delle gocce nell'intervallo micrometrico. Tra questi vantaggi vi sono la loro stabilità colloidale a lungo termine (le gocce più piccole non migrano all'interno della fase continua guidate dalla gravità o dagli effetti di galleggiamento), un migliore trasporto dermico e mucoso dei principi attivi, una migliore biodisponibilità e una maggiore estetica e sensibilità cutanea.

S1.1. Obbiettivi

Lo scopo di questo studio è duplice:

1. Il primo obiettivo generale è quello di esplorare la possibilità di produrre emulsioni o/w con gocce di dimensioni nanometriche mediante microfluidizzazione utilizzando una combinazione di CNCs e idrossipropilmetilcellulosa (HPMC), un tensioattivo derivato della cellulosa, che ha dimostrato di avere la capacità di modificare la bagnabilità dei CNCs (migliorando così la loro capacità di adsorbimento all'interfaccia olio/acqua).

Nell'ambito di questo progetto, il lavoro sperimentale è stato concepito per comprendere meglio il ruolo di HPMC come potenziatore delle prestazioni emulsionanti dei CNCs in termini di:

- Dimensione minima delle gocce raggiungibile
- Stabilità contro la scrematura
- Stabilità contro la coalescenza delle gocce.

Per affrontare i suddetti aspetti sono state preparate una serie di emulsioni di o/w (50% e 10% di olio) contenenti diverse quantità di CNCs e HPMC e sono state caratterizzate in termini di: distribuzione delle gocce in funzione del tempo (a t=0 e oltre) e velocità di scrematura delle gocce. Per definire il numero di emulsioni da preparare e caratterizzate per trarre conclusioni e analizzare tendenze statisticamente rilevanti relative alla stabilità, è stato utilizzato un approccio statistico di tipo sperimentale. Misurazioni reologiche e valutazioni della tensione interfacciale sono state effettuate su sistemi selezionati al fine di comprendere meglio le cause alla base di alcune delle tendenze osservate.

2. Il secondo obiettivo dello studio è stato quello di valutare le prestazioni delle emulsioni CNC/HPMC selezionate rispetto a quelle delle emulsioni stabilizzate con classici tensioattivi in base alla loro capacità di rilasciare un principio attivo come la luteina, un carotenoide di interesse per applicazioni topiche. Un sistema di diffusione standard come le *Franz-cells* (Vedi sezione 5.12.) è stato scelto per studiare il rilascio di luteina da diverse tipologie di emulsioni.

S2. Stato dell'arte

S2.1. Emulsioni di Pickering stabilizzate da CNCs

Tra i diversi tipi di particelle che sono stati studiati per la formulazione delle emulsioni Pickering, i CNCs hanno attirato l'interesse sia dei ricercatori accademici che degli sviluppatori di prodotti industriali grazie alle loro proprietà tecniche e alla loro biocompatibilità con la pelle umana. Diversi studi hanno descritto le prestazioni dei CNCs in relazione ai loro potenziali impieghi in formulazioni alimentari e farmaceutiche [29][13].

Il lavoro citato in questa sezione è solo una piccola parte della letteratura pubblicata sulle emulsioni di Pickering stabilizzate dai CNCs, ma comprende gli studi più importanti per comprendere gli obiettivi e i risultati di questo progetto. I CNCs sono stati testati come stabilizzatori di emulsioni sia da soli che in combinazione con co-stabilizzatori, come i derivati della cellulosa solubili in acqua o normali tensioattivi. Una panoramica della letteratura recente sulle emulsioni stabilizzate da CNCs è presentata nella *Tabella S1*.

Tabella S1. Sintesi della letteratura sulle emulsioni di Pickering stabilizzate da CNCs.

Agenti stabilizzanti	Sistema	Tecnica di emulsificazione	Principali risultati	Rif.
Nanocristalli di cellulosa (CNCs)	10 wt.% olio 0.05-2 wt.% CNC CNC/Oil = 0.45-18/100	Microfluidizzazione 600-1300 bar	Diminuzione della dimensione delle gocce con l'aumento della pressione Diminuzione della dimensione delle gocce con l'aumento della conc. di CNCs	[29]

			Minimo a 1200 bar, 0.75 wt.% CNC (d= 0.92 μm)	
Bromuro di didecildimetilammonio (DMAB) o bromuro di cetyltrimetilammonio (CTAB)	50 wt.% olio di dodecano 0.50 wt.% CNCs CNC/Oil = 0.5/100	Sonicazione	I tensioattivi adsorbiti cambiano la bagnabilità dei CNCs Maggiore concentrazione di tensioattivi, minore dimensione delle gocce e maggiore stabilità (d da ca. 40 a 8 μm)	[38]
CNC + metilcellulosa (MC) o idrossietil-cellulosa (HEC) o destrano (DEX)	50 wt.% olio di dodecano 0.1-1 wt.% CNCs CNC/Oil = 0.1-1-1/100 CNC/Polimero = 1/0,67	Sonicazione	Effetto sinergico di CNC + Polimero Nessun effetto sinergico per CNC + DEX CNC/Polimero = 1/0.05 (min. per saturare la superficie dei CNCs) Diminuzione della dimensione delle gocce con l'aumento della conc. Minimo a 1 wt.% CNCs (d= 2.8 μm per HEC e 1.9 μm per MC)	[39]
CNC + metilcellulosa (MC) o idrossietilcellulosa (HEC) + acido tannico (TA)	25 wt.% olio di mais 0.25 wt.% CNC CNC/Oil = 1/100 CNC/MC = 1/1	Sonicazione	Rinforzo dell'interfaccia delle gocce con acido tannico Emulsione ridispersa con la stessa dimensione delle gocce Importanza dell'ordine di miscelazione	[41]

Bai et al. [29] sono stati i primi a studiare la produzione di emulsioni di Pickering stabilizzate da solamente CNCs utilizzando la microfluidizzazione. Nel loro lavoro sono stati analizzati la stabilità e il diametro medio di emulsioni o/w contenenti 10 wt.% di olio a diverse concentrazioni di CNCs (0.05-2 wt.% della fase acquosa) e diverse pressioni di microfluidizzazione (600-1300 bar). La dimensione minima raggiungibile è risultata dipendere sia dalla pressione di microfluidizzazione che dalla concentrazione dei CNCs. Ad una pressione di microfluidizzazione costante di 900 bar, la dimensione media delle gocce dell'emulsione è diminuita fino a valori inferiori a 1 μm aumentando a concentrazioni di CNCs fino a 0.75 wt.%, rimanendo costante per valori superiori fino a 2 wt.%. L'aumento della concentrazione di CNCs ha favorito anche la stabilità contro la scrematura, sia per la riduzione delle dimensioni delle gocce che per un leggero aumento della viscosità della fase acquosa. Le pressioni di microfluidizzazione più elevate hanno favorito una riduzione del diametro delle gocce ad un valore minimo di 920 nm a circa 1200 bar. Un aumento inaspettato della dimensione delle gocce si è verificato a 1300 bar. Infine, sono stati testati diversi oli alimentari e tutti sono stati in grado di formare emulsioni stabili con i CNCs, compresi i trigliceridi a catena media (MCT) utilizzati in questo progetto. Questi sistemi sono rimasti stabili contro la coalescenza delle gocce in un ampio range di pH (3-10), concentrazioni di NaCl (≤ 100 mM) e temperature (30-90 °C) [29]. Il loro lavoro è il primo esempio che mostra la possibilità di produrre gocce di emulsione nanometriche stabilizzate dai CNCs utilizzando una tecnica di emulsificazione ad alta energia, come la microfluidizzazione [29], invece della sonicazione utilizzata nel loro lavoro precedente [40].

Tuttavia, sono state necessarie quantità relativamente elevate di CNCs (0.75-2%) per stabilizzare un'emulsione composta solo dal 10% di olio, il che, in termini di rapporto CNC/olio, significa che sono necessari 18 g di CNCs ogni 100 g di olio.

Nel secondo di una serie di due articoli, Bai et al. [37] hanno investigato la capacità di queste emulsioni stabilizzate da CNCs di modulare il rilascio di lipidi nel sistema digestivo umano. Monitorando gli acidi grassi liberi (FFA) in uno stadio dell'intestino tenue tramite un modello a 3 stadi del tratto gastrointestinale, è stato dimostrato che l'estensione finale degli FFA rilasciati è stata ridotta del 40%. Sulla base di questa constatazione, si ritiene che questi sistemi offrano possibilità molto interessanti per lo sviluppo di emulsioni per alimenti funzionali [37].

Il progetto presentato in questa tesi si basa sul lavoro di Hu et al. [38] che mostra che la capacità dei CNCs di stabilizzare le emulsioni può essere migliorata con l'aggiunta di co-stabilizzatori adsorbiti sulla superficie dei CNCs. Ad esempio, i CNCs, essendo anionici, potrebbero essere modificati *in situ* aggiungendo tensioattivi cationici come il DMAB e il CTAB [38]. Entrambi i tensioattivi adsorbono sui CNCs e modificano la bagnabilità delle particelle in base alla loro concentrazione. A basse concentrazioni di tensioattivi, le singole molecole si adsorbono con le code alchiliche che emergono dai CNCs rendendo le particelle più idrofobiche. A concentrazioni più elevate, al di sopra della concentrazione critica micellare, i tensioattivi adsorbono sulla superficie dei CNCs sotto forma di aggregati, con conseguente minore idrofobicità delle particelle. L'aggiunta di tensioattivi modifica la bagnabilità dei CNCs con conseguente maggiore stabilità delle emulsioni e riduzione della dimensione delle gocce da circa 40 μm a 8 μm. L'aumento della quantità di DMAB, che è il tensioattivo più idrofobico, ha mostrato anche la possibilità di stabilizzare emulsioni di tipo w/o. Infine, la modifica della superficie dei CNCs si è dimostrata una tecnica utile per aumentare sensibilmente le proprietà stabilizzanti dei CNCs [38]. Rispetto a Bai et al. [29], la stabilità di un'emulsione al 50% di olio viene raggiunta con solo lo 0.5% di CNCs, che in termini di rapporto CNC/olio è pari a 0.5 g per ogni 100 g di olio, 36 volte inferiore rispetto ai CNCs da soli.

Altri composti tensioattivi questa volta di origine naturale sono stati utilizzati per modificare la superficie dei CNCs. Derivati della cellulosa come l'idrossietilcellulosa (HEC) o la metilcellulosa (MC) sono stati studiati da Hu et al. per mostrare l'effetto di questi polimeri sulle proprietà delle emulsioni di Pickering stabilizzate da CNCs [39]. Sono state analizzate emulsioni con solo CNCs, con CNC+MC, con CNC+HEC, o con solo HEC o MC. Entrambi i polimeri si adsorbono efficacemente sulla superficie dei CNCs per raggiungere la copertura completa della superficie con un rapporto CNC/HEC o CNC/MC di 1/0.05 in massa. Emulsioni stabilizzate sia da CNC+HEC che CNC+MC, hanno mostrato una maggiore stabilità alla coalescenza e dimensioni delle gocce più piccole rispetto alle emulsioni stabilizzate da CNC, HEC e MC da soli, dimostrando l'effetto sinergico dell'adsorbimento di questi tensioattivi sui CNCs. Nel loro studio, le dimensioni delle gocce di emulsione più piccole sono state ottenute aumentando la concentrazione di CNCs, ad un rapporto costante tra CNC e polimero di 1/0.67. Calcolando la copertura superficiale delle goccioline d'olio dai CNCs per mantenere la stabilità, solo il 20% è risultato essere stabilizzato da CNCs modificati in superficie, mentre il restante 80% è stato stabilizzato solo da HEC o MC. Questo valore è notevolmente inferiore all'84% trovato da Capron et al. [40] necessario per ottenere

gocce stabili con CNCs non modificati, il che conferma ulteriormente che con i CNCs modificati superficialmente sono necessarie quantità di materiale inferiori per stabilizzare la stessa quantità di olio. Tuttavia, nel lavoro di Hu et al. [38] la dimensione delle goccioline di emulsione era ancora superiore a un micrometro, nessuna variazione del rapporto CNC/Polimero è stata studiata e la stabilità dei campioni è stata seguita per soli 6 giorni.

Le proprietà delle emulsioni CNC+HEC e CNC+MC sono state ulteriormente studiate dallo stesso gruppo di ricerca. Nel loro lavoro del 2016, Hu et al. [41] hanno investigato l'aggiunta di acido tannico alle emulsioni stabilizzate da CNC+HEC o CNC+MC e su come questo abbia permesso di liofilizzare e re-disperdere le emulsioni dopo l'essiccazione, mantenendo la stessa dimensione delle gocce durante tutto il processo, evitando così sostanzialmente la coalescenza delle gocce in condizioni di forte stress fisico. È stato dimostrato che l'acido tannico forma complessi colloidali con i polimeri HEC e MC rafforzando il guscio formato dai CNCs attorno alle gocce d'olio. Questo aumenta la stabilità durante la liofilizzazione, previene la coalescenza dopo l'essiccazione e consente la re-dispersione. Anche l'ordine di miscelazione dei diversi composti ha giocato un ruolo importante, poiché solo il campione in cui MC o HEC è stato miscelato con CNCs prima dell'emulsificazione con acido tannico poteva essere liofilizzato e re-disperso con successo [41].

S2.2. Emulsioni di Pickering come carrier di principi attivi

L'effetto delle emulsioni di Pickering come carrier di principi attivi risulta diverso a seconda del tipo di principio attivo studiato. In letteratura è stata studiata la somministrazione topica di farmaci idrofili in emulsioni w/o e lipofili in emulsioni o/w [31] [41] (*Tabella S2).* Purtroppo, nessuno studio sull'uso di emulsioni stabilizzate da CNCs come formulazioni di trasporto per la somministrazione di farmaci è stato trovato in letteratura.

Tabella S2. Sintesi della letteratura sulle emulsioni di Pickering nella somministrazione di farmaci.

Agenti stabilizzanti	Sistema	Principio attivo	Principali risultati	Rif.
Particelle di silice idrofobica	w/o 50 wt.% olio 1 % in peso di particelle di silice 0.9% di caffeina Membrana nelle Franz-cells: pelle di maiale Liquido ricettivo: 0.9% NaCl	Caffeina (idrofilo)	Migliore assorbimento cutaneo rispetto alla classica emulsione a base di tensioattivi, grazie alle specifiche interazioni della formulazione con le strutture cutanee, alla maggiore energia di adesione tra loro e alla penetrazione delle nanoparticelle nei primi strati dello strato corneo	[31]
Particelle di silice idrofobica	o/w 10 wt.% olio 7 wt.% di particelle di silice 0.1% retinolo Membrana nelle Franz-cells: pelle di maiale Liquido ricettivo: 1.5% Brij®98 e 0.5% acetato di alfa-tocoferolo	Retinolo (altamente idrofobo)	Nessuna permeazione cutanea dovuta all'elevata affinità della molecola con il mezzo lipidico dello strato corneo. Il retinolo si è fortemente trattenuto nello strato corneo e ha raggiunto l'epidermide e il derma in misura minore rispetto all'emulsione a base di tensioattivi	[41]
Amido	o/w 56 wt.% olio 200 mg amido/ml olio Celle di diffusione a	Salicilato di metile (idrofobo)	Penetrazione accelerata nel derma e attraverso tutta la pelle rispetto alle soluzioni standard di salicilato di metile	[42]

	flusso Membrana: pelle di maiale Liquido ricettivo: 0.1 M buffer fosfato			

Frelichowska et al. [31] hanno studiato la permeazione cutanea *in vitro* della caffeina in emulsioni stabilizzate da particelle di silice rispetto a emulsioni convenzionali stabilizzate da tensioattivi attraverso l'utilizzo di celle di diffusione (Franz-cells) con pelle di maiale come membrana. Entrambe le emulsioni presentavano la stessa composizione, dimensione delle gocce, frazione di volume della fase dispersa, viscosità, ma si differenziavano solo per la natura dell'agente stabilizzante, cioè per le diverse proprietà dello strato interfacciale olio/acqua. I risultati hanno mostrato un flusso di caffeina di 25 $\mu g\ cm^{-2}\ h^{-1}$ per l'emulsione di Pickering, tre volte superiore alle emulsioni classiche (9.7 $\mu g\ cm^{-2}\ h^{-1}$). Il maggiore assorbimento cutaneo della caffeina dall'emulsione di Pickering è dovuto, secondo gli autori, a specifiche interazioni della formulazione con le strutture cutanee, derivanti da una maggiore energia di adesione tra di esse, e alla penetrazione di nanoparticelle nei primi strati dello strato corneo, confermata da osservazioni al SEM che hanno rivelato la presenza di aggregati di silice fino a metà della profondità dello strato corneo (5 μm). L'adsorbimento di caffeina a questi aggregati di silice consiste in un meccanismo di trasporto supplementare per il farmaco attraverso la pelle.

Allo stesso modo della caffeina, il retinolo in emulsioni o/w è stato confrontato con una classica emulsione stabilizzata da comuni tensioattivi in test di penetrazione cutanea *in vitro* (Franz-cells con pelle di maiale) [41]. In questo caso, la quantità di retinolo permeata attraverso la pelle è stata trascurabile per entrambe le formulazioni, a causa dell'elevata affinità di questa molecola idrofoba con il mezzo lipidico dello strato corneo. Inoltre, anche la quantità di retinolo presente nella pelle era identica per entrambi i sistemi. Ciò che è risultato diverso è stato, in primo luogo, il forte accumulo del retinolo dell'emulsione di Pickering nello strato corneo rispetto all'emulsione a base di tensioattivi. In secondo luogo, il retinolo immagazzinato all'interno dello strato corneo durante le 24 ore di esposizione della formulazione con la pelle è stato poi rilasciato lentamente nelle successive 24 ore dopo la rimozione della formulazione.

Le considerazioni fatte per il retinolo non possono essere generalizzate per tutti i composti lipofili in quanto è stato dimostrato che principi attivi meno idrofobici stabilizzati in emulsioni di Pickering hanno mostrato, al contrario, una penetrazione accelerata nel derma e attraverso tutta la pelle [42].

S3. Approccio

L'intero progetto è diviso in due macro-sezioni: lo studio di formulazione e lo studio sul rilascio del principio attivo.

Nella prima sezione la composizione della formulazione è stata ottimizzata in termini di concentrazioni di CNCs e HPMC per poter raggiungere il minimo diametro delle gocce possibile e la miglior stabilità contro la scrematura e la coalescenza. La concentrazione dei CNCs è stata variata da 0.03 a 0.75 wt.% della fase acquosa. Nel frattempo il rapporto CNC/HPMC (wt./wt.) è stato modificato da 1/0.05 a 1/1, il che significa che la quantità di HPMC variava da 0.005 a 0.75 wt.% della fase acquosa. Risulta importante notare che a questi valori del rapporto CNC/HPMC variare la quantità di HPMC nel sistema significa variare l'eccesso di questa molecola nella fase acquosa dell'emulsione e non il grado di adsorbimento della superficie dei CNCs.

Le emulsioni sono state formulate tramite microfluidizzazione ad una pressione di 500 bar e un numero di passaggi attraverso il microfluidizzatore pari a 5. Il diametro medio delle emulsioni ($D_{4/3}$) è stato misurato tramite laser light scattering via un Mastersizer 2000 (Malvern Instruments), mentre la velocità di scrematura è stata calcolata tramite un analizzatore a scansione ottica Turbiscan (Formulaction) (Vedi sezione 5.11.).

La scelta di quali esperimenti effettuare è stata effettuata tramite un approccio statistico di tipo sperimentale utilizzando il software MODDE Pro v. 12.1 (Umetrics) che ha prodotto una matrice di 12 esperimenti (Vedi sezione 5.6.).

Per lo studio sul rilascio del principio attivo sono state utilizzate le Franz-cells con membrana sintetica per poter comparare il rilascio della luteina tra due emulsioni stabilizzate da CNCs e HPMC con diverso diametro delle gocce e tra una emulsione CNC/HPMC e una emulsione classica stabilizzata da una comune lecitina della soia (Lipoid S100) avente le stesse dimensioni delle gocce.

S4. Risultati e discussione

S4.1. Studio di formulazione

S4.1.1. Dimensione goccia a t = 0

L'aumento della concentrazione di CNCs ha favorito la riduzione delle dimensioni delle gocce dell'emulsione, come riportato in letteratura. Più interessante è stato notare come anche un aumento della concentrazione di HPMC nel sistema abbia causato lo stesso effetto.

La ragione della diminuzione delle dimensioni delle goccioline quando si aumenta la concentrazione di CNCs è, all'inizio, abbastanza intuitiva. Più CNCs significa più materiale per poter stabilizzare un'area interfacciale più ampia. Tuttavia, questo ragionamento non è sempre valido. È vero quando la mancanza di emulsionante è l'unico ostacolo che impedisce la stabilizzazione di gocce più piccole. Avere una quantità sufficiente di CNCs è essenziale per evitare che le goccioline appena formate coalescano subito dopo la microfluidizzazione. D'altra parte, se non è stato possibile formare gocce più piccole al passaggio della emulsione all'interno del microfluidizzatore l'aumento di CNCs non influenzerà il processo di rottura delle gocce e le loro dimensioni non vedranno alcun cambiamento.

Quello che invece può favorire una più efficacie rottura delle gocce nel microfluidizzatore è la presenza di un tensioattivo che riduca la tensione superficiale e quindi favorisca la rottura dell'interfaccia e allo stesso tempo aumenti anche la viscosità della fase acquosa in modo da rendere più efficiente il trasporto del momento all'interno della camera di microfluidizzazione. HPMC risulta essere in grado di fornire entrambe queste proprietà al sistema ed è proprio per questo motivo che un aumento della sua concentrazione all'interno del sistema risulta in una diminuzione del diametro delle gocce. Inoltre, la capacità di HMPC di diffondere all'interfaccia olio-acqua molto più rapidamente dei CNCs aiuta nel prevenire che le gocce appena formate vadano a coalescere per la mancanza di stabilizzatore nelle vicinanze.

Purtroppo, la dimensione delle gocce di tutti e 12 le emulsioni al 50% di olio è risultata essere al di sopra di 1 micron, con un massimo di 152 μm e un minimo di 1.68 μm (ad alte concentrazioni di CNCs e HPMC).

Il motivo del mancato raggiungimento delle dimensioni nanometriche può risiedere o nella difficoltà del sistema di rompere le gocce dell'emulsione in goccioline più piccole o nella scarsa disponibilità di emulsificante presente per poter prevenire la coalescenza immediata delle gocce all'interno della camera di microfluidizzazione. Per poter rispondere a questo enigma è stato utile calcolare quale fosse la quantità necessaria di CNCs per poter stabilizzare le gocce d'olio. Da letteratura, è noto che almeno il 20% della goccia deve essere coperta da CNCs per poter prevenire immediata coalescenza [39]. Calcolando tale valore per le emulsioni con 50% di olio (Vedi *Table 10*) si è potuto notare come nei casi con il minimo diametro di goccia, ovvero ad alte concentrazioni di CNCs e HPMC, questo si avvicini sensibilmente al valore del 20% riportato in letteratura. Questo potrebbe far pensare che ci sia una mancanza di CNCs nel sistema per poter stabilizzare gocce con diametri minori. Per poter confermare questa tesi si potrebbe suggerire di aumentare la quantità di CNCs nel sistema mantenendo lo stesso rapporto CNC/HPMC. Tuttavia, ciò non è fisicamente fattibile in quanto la presenza sia di CNCs che di HPMC ad alta concentrazione provoca l'intasamento della camera del

microfluidizzatore, a causa dell'alta viscosità. Per questo motivo si è deciso di effettuare una diminuzione della quantità di olio, in modo da poter mantenere la stessa concentrazione di CNCs e HPMC (e lo stesso rapporto), ma con un rapporto CNC/olio molto più alto.

Formulando le stesse emulsioni ma con una percentuale di olio del 10% la tesi è stata confermata raggiungendo un diametro di 588 nm.

S4.1.2. Stabilità

S4.1.2.1. Coalescenza

Insieme alla dimensione delle gocce, la stabilità dell'emulsione contro la coalescenza è importante per formulare un prodotto che possa essere di interesse per il mercato.

Le emulsioni stabilizzate da CNCs sono comunemente note per essere stabili contro la coalescenza grazie al loro forte adsorbimento all'interfaccia delle gocce d'olio e alla loro repulsione sterica ed elettrostatica. Questo è confermato dal fatto che un aumento della concentrazione di CNCs diminuisce la deviazione standard dei diametri dell'emulsione nel tempo (Vedi *Figure 30*), il che significa un aumento della stabilità dell'emulsione (Vedi sezione 5.7.). Quando i CNCs sono in alta concentrazione, ma la concentrazione di HPMC è bassa, non sono stati osservati cambiamenti sostanziali nel diametro medio e nella sua distribuzione dopo due mesi (bassa deviazione standard).

Al contrario, quando sia le concentrazioni di CNCs che quelle HPMC sono basse la deviazione standard risulta essere alta e i sistemi in quella regione coalescono con uno spostamento a diametri maggiori dell'intera distribuzione delle dimensioni della goccia (Vedi *Figure 32*).

Una piccola deviazione standard si ottiene anche nel caso in cui sia le concentrazioni di CNCs che quelle HPMC siano elevate. Se si considerano le distribuzioni delle dimensioni delle gocce, si può notare che alcune perturbazioni si verificano a diametri più elevati. Gocce con diametro maggiore sembrano essere rilevate dallo strumento e la loro presenza sembra crescere nel tempo (Vedi *Figure 33*).

Due sono le spiegazioni possibili per questo fenomeno. Uno è che la grande quantità di HPMC nel sistema stabilizzi da sola un piccolo numero di gocce. Non essendo HPMC un buon stabilizzatore, questo potrebbe portare a coalescenza nel tempo, che però non creerebbe uno spostamento dell'intera distribuzione. D'altra parte, l'elevata presenza di HPMC nel sistema potrebbe facilitare l'aggregazione di piccole gocce in cluster più grandi, che non vengono rotti dal mixer presente nel Mastersizer prima della misurazione. Per valutare quale delle due ipotesi potesse essere più probabile, le emulsioni sono state visualizzate con un microscopio ottico (ZEISS Microscopy) o vorticate, ma non è stato possibile poter concludere con sicurezza quale dei due fenomeni fosse il responsabile di tale effetto.

S4.1.2.2. Scrematura

L'effetto della variazione della concentrazione di CNCs e HPMC sulla stabilità contro la scrematura è stato studiato analizzando la velocità di scrematura delle emulsioni (Vedi sezione 5.11.).

Sia l'aumento della concentrazione di CNCs che di HPMC riducono la velocità di scrematura, ovvero migliorano la stabilità contro questo fenomeno. La spiegazione si collega alla legge di Stokes mostrata nell'*Equazione S1*.

$$v = \frac{g}{18\ \mu_{cont.ph.}} * \left(\rho_{drop} - \rho_{cont.ph.}\right) * D_{drop}^{2}$$

Equazione S1. La legge di Stokes.

Tra le diverse formulazioni, sia la viscosità della fase acquosa ($\mu_{cont.ph.}$) che la dimensione delle gocce (D_{drop}) cambiano. Come si può vedere nell'*Equazione S1*, i due contribuiscono a modificare la velocità di scrematura. Maggiore è la viscosità, più lenta è la crematura, ma più grande è la dimensione delle gocce, più veloce è la crematura. Ad esempio, i bassi tassi di scrematura ad alte concentrazioni di CNCs e HPMC (Vedi *Figure 36*) possono essere spiegati dall'alta viscosità della fase acquosa e dalle piccole dimensioni delle gocce. Al contrario, le alte velocità di scrematura a basse concentrazioni di CNCs e HPMC (Vedi *Figure 36*) sono causati dalla bassa viscosità della fase acquosa e dalle grandi dimensioni delle gocce.

S4.1.3. Formulazione finale per lo studio sul rilascio di un principio attivo

Le alte concentrazioni di CNCs e HPMC hanno dimostrato di favorire sostanzialmente la riduzione delle dimensioni delle gocce e la stabilità contro la scrematura. Tuttavia, le elevate concentrazioni di HPMC sembrano fornire un effetto negativo sulla distribuzione dei diametri delle gocce delle emulsioni, indipendentemente dal fatto che si tratti di coalescenza o di formazione di agglomerati. La presenza di agglomerati ridurrebbe la potenziale area interfacciale delle gocce d'olio contenenti il principio attivo con la pelle e quindi non sono auspicabili per le formulazioni cosmetiche/farmaceutiche.

Per questo motivo è stato condotto un ulteriore esperimento con l'obiettivo di ottenere una dimensione delle gocce simile a quella minima ottenuta per le emulsioni al 50% di olio (1.68 µm), ma anche una distribuzione delle gocce che potesse essere stabile nel tempo. Per fare ciò, la concentrazione di HPMC è stata ridotta dallo 0.75% allo 0.3%, il che ha permesso di raddoppiare la concentrazione di CNCs all'1.5% (non si è verificato alcun intasamento del microfluidizzatore). Grazie alla possibilità di utilizzare una pressione di microfluidizzazione superiore, pari a 900 bar, si è potuto ottenere una emulsione con un diametro di 2.67 µm e una distribuzione stabile per almeno un mese. L'aumento della pressione insieme all'aumento della concentrazione CNCs ha creato un doppio effetto. Una pressione più elevata ha significato un maggiore apporto di energia al sistema per compensare la mancanza di HPMC a favore della rottura delle gocce. Inoltre, una maggiore concentrazione di CNCs ha causato un aumento della disponibilità dello stabilizzatore e della sua vicinanza alle goccioline appena formate.

S4.2. Studio sul rilascio di un principio attivo

S4.2.1. Configurazione del metodo analitico (Franz-cells)

Una buona parte di questo progetto è stata spesa nell'ottimizzazione della configurazione delle Franz-cells per poter avere un metodo analitico che potesse garantire la permeazione della luteina dalla formulazione attraverso la membrana al fluido ricettivo e allo stesso tempo non permettesse la migrazione del fluido ricettore verso il lato donatore. Ciò ha comportato il test di diverse combinazioni di membrane, composizioni di fluidi ricettivi e concentrazioni di luteina nella formulazione.

Una descrizione della configurazione finale utilizzata è riportata nella *Tabella S3*.

Tabella S3. Configurazione finale Franz-cells.

Membrana	Idrofila Polietersulfone Dimensione dei pori: 6 nm
Fluido del recettore	70% EtOH 2% Tween80

S4.2.2. Confronto tra emulsioni di Pickering CNC-HPMC con diverse dimensioni di goccia

La dimensione delle gocce è un fattore chiave per regolare la permeabilità di un attivo attraverso una membrana [30]. Dimensioni più piccole delle gocce significano una maggiore area interfacciale disponibile per il principio attivo per passare dalle goccioline d'olio attraverso la membrana nel fluido ricettore. Questo è confermato dai risultati del confronto tra due emulsioni uguali per composizione me con diverso diametro delle gocce. La percentuale di luteina permeata attraverso la membrana è notevolmente superiore, più che doppia, dopo 24 ore, per l'emulsione con un diametro di circa 3 μm (11%) rispetto a quella con un diametro di 40 μm (5%). Questa potrebbe essere una validazione di ciò che è stato mostrato in letteratura, evidenziando l'importanza di raggiungere diametri sempre più piccoli nelle emulsioni utilizzate per la somministrazione topica.

S4.2.3. Confronto con un'emulsione classica

L'emulsione CNC-HPMC è stata anche confrontata con un'emulsione stabilizzata da un comune tensioattivo, una lecitina della soia (Lipoid S100). Le due emulsioni presentano lo stesso diametro delle gocce e la stessa concentrazione di principio attivo. Fin dalle prime ore la percentuale di luteina permeata attraverso la membrana è più alta per l'emulsione classica rispetto all'emulsione CNC-HPMC, raggiungendo dopo 24 ore valori del 19% rispetto al 11% dell'emulsione CNC-HPMC. La presenza di un monostrato denso di nanoparticelle all'interfaccia olio/acqua potrebbe fungere da barriera che regola il rilascio del principio attivo in fase acquosa. Questo potrebbe essere utile nelle formulazioni topiche che richiedono un rilascio controllato o prolungato di farmaci. Tuttavia, conclusioni definitive sono difficili da trarre per questo sistema, in quanto la differenza di viscosità tra le due emulsioni è tale da non

poter escludere che lo stadio controllante sia il trasporto diffusivo delle gocce d'olio dal bulk dell'emulsione alla membrana.

S5. Conclusioni

S5.1. Studio di formulazione

- Emulsioni di o/w con gocce di dimensioni nanometriche (diametro medio delle gocce di circa 600 nm) sono state formulate mediante microfluidizzazione utilizzando una combinazione di CNCs e idrossipropilmetilcellulosa (HPMC). Le più piccole dimensioni di goccia di emulsione sono state ottenute utilizzando una pressione di microfluidizzazione di 500 bar con un sistema contenente il 10% di olio, 0.55% CNCs e 0.55% HPMC (si tratta di un rapporto CNC/HPMC di 1/1 e un rapporto CNC/olio di 5/100). In queste condizioni di concentrazione, la miscela acquosa iniziale di emulsionante consisteva sia di CNCs modificati con HPMC che di HPMC in eccesso.

- All'interno del range di concentrazione di CNCs e HPMC studiato (da 0.03% a 1.5% CNCs e da 0.005% a 0.75% HPMC), le alte concentrazioni di CNC in combinazione con le alte concentrazioni di HPMC hanno favorito una riduzione della dimensione della goccia. Per una data pressione di microfluidizzazione e un rapporto fisso CNC/olio le più piccole dimensioni di goccia di emulsione sono state raggiunte con una concentrazione di CNCs di 0.55-0.75% e un rapporto CNC/ HMPC di 1/0.67-1 (cioè con largo eccesso di HPMC). L'eccesso di HPMC nel sistema favorisce la formazione di piccole gocce di emulsione grazie alla sua capacità di abbassare la tensione interfacciale all'interfaccia olio-acqua e di aumentare la viscosità della fase continua dell'emulsione. Allo stesso tempo, nel sistema CNC-HPMC una quantità minima di CNCs (0.55%) si è rivelata essenziale per consentire la stabilizzazione delle gocce appena formate ed evitare l'immediata coalescenza delle gocce.

- La capacità di raggiungere dimensioni nanometriche a pressioni di microfluidizzazione fisse è modulata dalle variazioni del rapporto CNC/olio. Data l'elevata viscosità delle emulsioni con alte concentrazioni di CNCs e HMPC oltre concentrazioni dello 0.75%, una variazione del rapporto CNC/olio è stata effettuata cambiando le quantità relative di olio e acqua nell'emulsione piuttosto che aumentando la concentrazione di CNCs e HPMC nella fase acquosa. Mentre la dimensione minima raggiungibile della goccia con un rapporto CNC/olio di 0.75/100 (emulsione contenente il 50% di olio) era di 1.68 μm, emulsioni con dimensioni medie di circa 600 nm sono state raggiunte con la stessa concentrazione di CNCs e con lo stesso rapporto CNC/HPMC, quando il rapporto CNC/olio era di 7/100 (emulsione contenente il 10% di olio).

- La presenza di un eccesso di HPMC in fase acquosa (concentrazioni di HPMC ≥ 0.3%) ha favorito la stabilità delle emulsioni contro la scrematura, per l'effetto combinato di minori dimensioni delle gocce e maggiore viscosità.

- Le alte concentrazioni di CNCs (0.75%) e le basse concentrazioni di HPMC (< 0.55%), hanno mostrato una buona stabilità verso la coalescenza per un periodo di almeno 2 mesi senza variazioni nella distribuzione delle dimensioni della goccia nel tempo. Alte concentrazioni di HPMC (0.55-0.75%) hanno mostrato, tuttavia, o la coalescenza di una piccola parte delle gocce, probabilmente quelle stabilizzate solo da HPMC, o l'agglomerazione di piccole gocce in cluster più grandi.

S5.2. Studio sul rilascio di un principio attivo

- La percentuale di luteina permeata attraverso la membrana negli esperimenti di diffusione tramite le Franz-cells è stata dopo 24 ore 11% per un'emulsione CNC-HPMC (diametro di ca. 3 μm) e 5% per la stessa emulsione non microfluidizzata (diametro di 40 μm). La differenza nella dimensione delle gocce ha permesso un più rapido rilascio del principio attivo, grazie ad una maggiore superficie disponibile tra le gocce e la membrana.
- L'emulsione classica ha mostrato una percentuale di luteina nel fluido recettore dopo 24 ore del 19%, 1.7 volte di più rispetto al caso della soluzione CNC-HPMC con la stessa quantità di luteina e simili dimensioni della goccia. Ciò potrebbe confermare il ruolo dello strato di CNCs all'interfaccia olio-acqua che funge da barriera, regolando il rilascio di luteina dalle gocce d'olio in quello che sembra essere un rilascio più sostenuto. Tuttavia, la differenza di due ordini di grandezza nella viscosità delle due emulsioni non permette di trarre conclusioni definitive.

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Formulation of Nanoemulsions Stabilized by Cellulose Nanocrystals

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1. Table of contents

Abstract

Cellulose nanocrystals (CNCs) are bio-based nanoparticles with the ability to stabilize oil and water emulsions thanks to their intermediate wettability and nanometric size. These and other types of particle-stabilized emulsions, commonly referred to as Pickering emulsions, are of great academic and industrial interest due to their superior stability against drop coalescence compared to classical surfactant-stabilized emulsions. In addition, the presence of a densely packed layer of particles at the oil-water interface is expected to impact the encapsulation ability of the emulsion droplets opening up for the possibility to use these systems to modulate the release of active substances in the context of oral or topical delivery formulations used in pharmaceutical and cosmetic applications. In these types of applications, the use of emulsions with nano-sized drops is advantageous due to their long-term colloidal stability, improved dermal and mucosal transport of actives, improved bioavailability and greater aesthetic appeal and skin feel.

This study had two main objectives. The first one was to explore to possibility to produce o/w emulsions with submicron-size drops by means of microfluidization using a combination of CNCs and hydroxypropyl methylcellulose (HPMC), a surface-active cellulose derivative that has been shown to have the ability to modify the wettability of CNCs (thereby enhancing their ability to adsorb at the oil/water interface). An important aspect of this first part of the study also involved gaining better understanding on the separate contributions of CNCs and HPMC to the properties of the resulting emulsions. The second objective of the work was to assess the performance of selected o/w CNC/HPMC compared to that of surfactant-stabilised emulsions in terms of their ability to deliver lutein, a hydrophobic prototype active of interest for topical delivery applications.

For the first part of this study, a series of o/w emulsions (50 and 10% w oil) containing different amounts of CNCs and HPMC were prepared and characterised in terms of: drop-size distribution after (at t=0 and over time) as well as in terms of the creaming rate of the drops. A design of experiments statistical approach was used to define the number of emulsions systems to be prepared and characterised. For the lutein delivery study, an in vitro permeation test via Franz-cells using a synthetic membrane was used to study the release of lutein from different emulsion formulations.

It was possible to produce o/w emulsions with submicron-size drops (average drop diameters down to ca. 600 nm) by means of microfluidization using a combination of CNCs and hydroxypropyl methylcellulose (HPMC). Within the CNCs and HPMC concentration range studied (0.03% to 1.5% CNC and 0.005% to 0.75% HPMC), high concentrations of CNCs in combination with high concentrations of HPMC favoured a reduction of the drop size. However, the ability to attain submicron drop sizes at fixed microfluidization pressures was found to be modulated by changes in the CNCs to oil ratio on the system. While the minimum attainable drop size at CNCs to oil ratio of 0.75:100 (emulsion containing 50% oil) was 1.68 μm, emulsions with average sizes of ca. 600 nm were attained at the same CNC concentration and CNC/HPMC ratio when the CNCs to oil ratio was 7:100 (emulsion containing 10% oil).The presence of excess of HPMC in the aqueous phase (HPMC concentrations ≥ 0.3%) favoured the stability of the emulsions against creaming, due to the combined effect of having smaller drop sizes and higher continuous phase viscosities. Emulsions with high concentrations of CNCs (0.75%) and low HPMC concentrations (< 0.55%), showed good stability towards coalescence for a period of 2 months with no change in drop size distribution over time.

The amount of permeated lutein after 24 hours from a CNC/HPMC emulsion containing 1% of lutein was found to be ca. twice as high (11% vs. 5%) in emulsions with a median drop size of 3 μm compared to emulsions with 40 μm drops. At the same time, the total permeated amount of lutein from emulsions stabilized with soy lecithin was found to be higher (19% vs. 11%) than that from CNC/HPMC emulsions with similar drop sizes. This difference could be due to the regulating effect of the densely packed CNC-HPMC interfacial layer but could also be related to the much lower viscosity (5 vs. ca. 500 mPa.s at 100^{-1} s) of the lecithin emulsions.

2. Introduction

Cellulose nanocrystals (CNCs) are a new class of commercially available nanoparticles most commonly derived from wood. Due to their "bio-derived" status and their functional properties there is a great interest to use them in the context of commercial applications in particular in cosmetic and personal care products. In recent years, a number of studies have reported on the ability of CNCs to stabilize emulsions thanks to their intermediate wettability and nanometric size, which allow them to adsorb at the oil-water interfaces enabling emulsion stability by the so-called Pickering mechanism. Pickering emulsions are of particular academic and industrial interest. This is due to the fact that, compared to emulsions stabilized by common small molecule emulsifiers, Pickering emulsions are known to display enhanced stability towards droplet coalescence and Ostwald ripening. In addition, the existence of a densely packed layer of particles at the droplet interface will impact the encapsulation ability of the emulsions drops, opening the possibility to use these systems in controlled release applications.

CNCs have been reported to have the ability to stabilise oil in water (o/w) emulsions, while CNCs whose wettability has been tuned by the adsorption of a surface active species have been shown to have the ability to stabilise both o/w and w/o emulsions (depending on the wettability). In general, the results available in the literature indicate that compared to emulsions stabilised solely by CNCs, good emulsion stability can be attained at lower overall CNC concentrations in emulsions produced by CNCs whose wettability has been modified by adsorption of surface-active species. Regardless of the nature of the continuous and dispersed phase (o/w or w/o emulsions), most studies describing CNC-stabilised emulsions deal with emulsions with droplet sizes in the micron size range. Only very recently, the possibility to make sub-micron droplet size CNC-stabilised emulsions was demonstrated. This was achieved through the use of microfluidisation, a well-established, high-pressure emulsification technique. The sub-micron o/w emulsions were stabilised solely by CNCs and contained 10% of oil and CNC concentrations of 0.75 wt.% in the aqueous phase [29].

In the context of nutraceutical, cosmetic and pharmaceutical applications, emulsions with average drop sizes in the nanometer size range have been reported to display a number of advantages compared to their counterparts with drop sizes in the micrometer range. Among these advantages are their long-term colloidal stability (smaller droplets do not migrate within the continuous phase driven by gravity or buoyancy effects), improved dermal and mucosal transport of actives, and improved bioavailability and greater aesthetic appeal and skin feel.

2.1. Objectives

The aim of this study is twofold:

3. The first overall goal is to explore to possibility to produce o/w emulsions with submicron-size drops by means of microfluidisation using a combination of CNCs and hydroxypropyl methylcellulose (HPMC), a surface-active cellulose derivative that has been shown to have the ability to modify the wettability of CNCs (thereby enhancing their ability to adsorb at the oil/water interface).

   In the context of this project, the experimental work was designed to gain better understanding on the role of HPMC as an enhancer of the emulsifying performance of CNCs in terms of:

   - Minimum attainable droplet size
   - Stability against creaming

- Stability against drop coalescence.

To address the above-mentioned aspects a series of o/w emulsions (50% and 10% oil) containing different amounts of CNCs and HPMC were prepared and characterised in terms of: drop-size distribution as a function of time (at t=0 and over time) and the creaming rate of the drops. A design of experiments statistical approach was used to define the number of emulsions that needed to be prepared and characterised to draw statistically relevant conclusions and trends related to stability. Rheological behaviour and interfacial tension assessments were made on selected systems in order to gain better understanding of the underlying causes behind some of the tendencies observed.

4. The second goal of the study was to assess the performance of selected o/w CNC/HPMC emulsions compared to that of surfactant-stabilised emulsions based on their ability to deliver lutein, a hydrophobic prototype active of interest for topical delivery applications. A Franz cell set-up using a synthetic membrane to support the formulation, was used to study the release of lutein from different emulsion formulations.

3. Theoretical background

3.1. Cellulose nanomaterials (CNMs)

Nanoparticles are of great interest today due to the unique characteristics associated with their nanoscale size, morphology, and large surface area. Among different materials, such as silica, other oxides and clays, cellulose is also considered to be a promising sustainable alternative in many industrial applications. CNMs is a generic term that refers to nanometer-scale crystalline particles that are extracted from natural sources of cellulose, such as wood and cotton. This category broadly includes three different types of CNMs: cellulose nanocrystals (CNCs), cellulose nanofibrils (CNFs), and bacterial cellulose (BC) [1]. The difference between these cellulosic materials mainly resides in differences in particle size, size distribution and morphology, which are related to the extraction/production method that is employed. In this report, the focus is on a commercial grade of CNCs produced from Canadian bleached softwood.

3.1.1. Cellulose nanocrystals (CNCs)

3.1.1.1. Production method

Cellulose is the most abundant polymer in the world [2]. However, it does not occur in nature as an isolated individual molecule [3]. In wood, for example, which is a common cellulose source to produce CNCs, cellulose fibers form together with lignin and hemicelluloses a complex and robust structure, creating the wood matrix shown in *Figure 1*. Due to this heterogenous composition several mechanical and/or chemical treatments are required to isolate crystalline cellulose elements, such as CNCs, from wood.

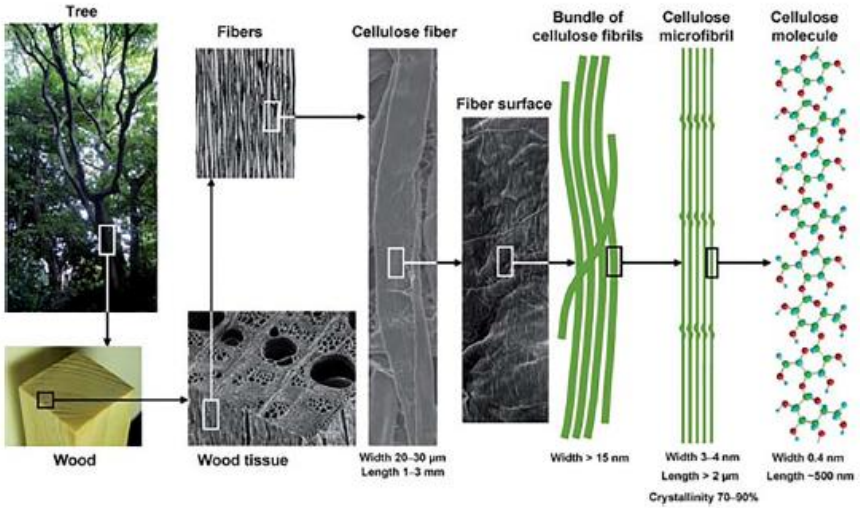

Figure 1. Schematic of wood structure and molecular composition [4].

The mechanical and/or chemical treatments required to isolate cellulose from wood can be divided into two main steps: pre-treatment and refinement. The first is meant to homogenize

and purify the starting material to make it react more consistently in subsequent treatments. In this phase cellulose needs to be separated from non-cellulosic material such as hemicelluloses, lignin, fats and waxes, proteins, and inorganic contaminants. A well-established technique to achieve this is pulp production in pulp mills. Once cellulose has been isolated, its fibre structure needs to be broken down to extract the crystalline regions. For CNCs, the primary method is via sulfuric acid hydrolysis which has the benefit of grafting negatively charged sulfate half-ester groups on the CNC surfaces which provide colloidal stability in aqueous suspension [4]. General operating conditions of this method are an acid concentration between 60- 65 wt.%, a temperature approximately around 45-55 °C and a hydrolysis time between 30 and 90 minutes [5]. Moreover, the concentration of the acid, temperature and time of the process have been shown to alter the quality of the final CNCs, such as their length and crystallinity [6].

When talking about CNMs it is important to have in mind the difference between CNCs and CNFs, which arises from the use of different isolation methods to isolate the CNM. CNCs are produced typically by chemical treatments, usually sulfuric acid hydrolysis, that liberate the short, rod-like crystalline sub-units from within the cellulose microfibril, followed by an ultrasonication treatment. Whereas longer CNFs are predominately produced by strong mechanical treatment (e.g. high pressure microfluidization or grinding), usually preceded by a chemical or enzymatic pre-treatment to lessen the energy requirement of the mechanical treatment, yielding a broad family of materials under the CNF umbrella, depending on material source and details of the preparatory route. CNF and CNCs have different particle morphologies and sizes, as is shown in *Figure 2*, and often also different surface chemistries, which is the reason why CNCs and CNFs may interact/respond to a given environment or application in different ways [1].

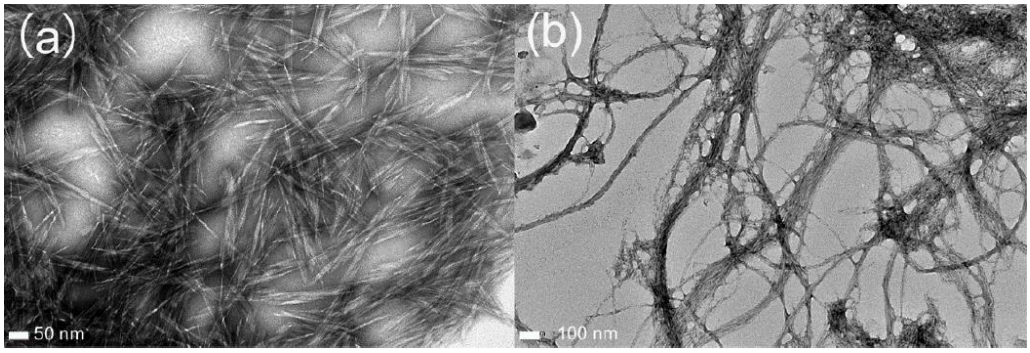

Figure 2. TEM images of (a): CNC and (b): CNF [4].

3.1.1.2. Properties of CNCs

As seen in *Figure 2a,* CNCs are rod-shaped nanoparticles with a large aspect ratio. Their typical dimensions are 50–350 nm in length, 5–20 nm in width, and an aspect ratio of 5–30 [6]. This variation in particle aspect ratio, as well as variations in surface chemistry and charge, are determined by the hydrolysis conditions and cellulose source. Depending on which type of acid is used, different charged groups are present on the surface of the CNCs. Sulfuric acid produces anionic sulfate half-esters, meanwhile the result of using phosphoric acid are anionic phosphate half-esters. Commercially, CNCs from sulfuric acid hydrolysis can be found in suspensions (6-12 wt.%) or in re-dispersible freeze/spray-dried powders. In

Figure 3, the acid form of the CNCs can be seen, where the counterion of the sulfate half-esters groups (-O-SO_3^-) is H^+. However, CNCs are always sold in the neutralized sodium-salt form, where it is Na^+ that acts as the counterion to the charged groups on the surface of the nanocrystals [1].

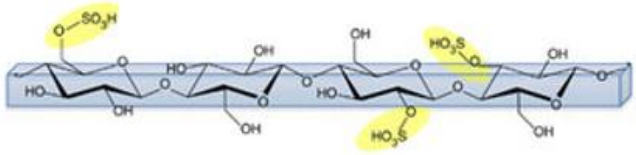

Figure 3. Schematic of anionic CNCs with sulfate half ester surface groups [7].

The key properties of CNCs are their high-surface area, availability, biodegradability and impressive mechanical strength. A Young´s modulus of around 150 GPa, higher than Kevlar, makes this material an ideal reinforcing agent for nanocomposites [3]. Moreover, its intermediate hydrophilicity is an interesting characteristic in terms of its possible use as stabilizing agent in emulsions, foams and gels. Being a biocompatible, non-toxic material opens the possibility to use it as a safe stabilizer in food-grade and pharmaceutical formulations [29]. Here, the main advantages of using CNCs compared to other CNMs are less polydisperse sizes, better morphology control, and defined surface properties and crystalline planes, which allow for more reproducible formation of emulsion droplets [8]. Finally, the presence of hydroxyl groups on CNM surface provides sites for selective modification and tuning of the final properties.

3.2. Hydroxypropyl methylcellulose (HPMC)

Hydroxypropyl methylcellulose (HPMC) is a polymer that belongs to the family of cellulose ethers, where the hydroxyl groups present in the cellulose rings or anhydroglucose units, are replaced by both methoxy (MeO) and hydroxypropyl groups (HP), as can be seen in *Figure 4*.

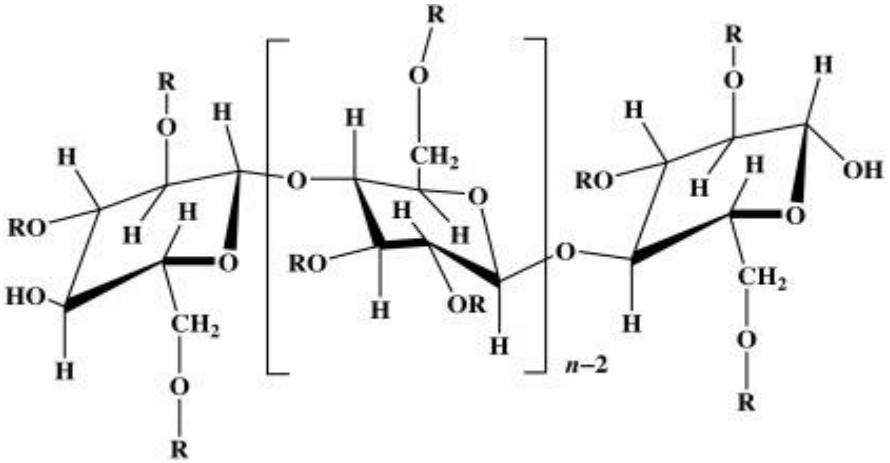

Figure 4. Chemical structure of hydroxypropyl methylcellulose, R = H, −CH3 or -CH2CH(OH)CH3 [9].

To obtain HPMC, cellulose is heated with a caustic solution and mixed with both methyl chloride and propylene oxide. The etherification reaction reduces the amount of hydroxyl groups in the system and therefore limits the strong hydrogen bonding tendencies of cellulose to itself, resulting in a solvent-soluble compound. Usually, the amount of the functional group

substitution is described as a weight percent (%) or degree of substitution which is the average number of hydroxyl groups substituted by MeO or HP per cellulose anhydroglucose unit. Commercially, polymers with different degrees of polymerization are present, i.e. different viscosity and molecular weight. HPMC polymers have unique thermogelation properties, gelling at temperatures in the range of 75-90 °C. The relatively hydrophobic MeO and HP groups give amphiphilicity and surface activity to the polymers and make them suitable for reducing surface/interfacial tension. Thanks to these characteristics and its biodegradable and biocompatible nature, HPMC has a wide range of applications in drug delivery, dyes and paints, cosmetics, adhesives, coatings, agriculture, and textiles [10–12]. In this work, HPMC will be used to functionalize the CNCs through adsorption and enhance their hydrophobicity and surface activity [13].

3.3. Emulsions

An emulsion is a dispersion of one liquid in another liquid. Being a dispersion and not a solution it is evident that the two liquids must be immiscible. In such systems at least two phases are present, which are normally referred to as the continuous or external phase and the dispersed or internal phase. The majority of the emulsions consist of water as one phase and an organic fluid as the other phase.

The latter is normally referred to as the “oil phase” even when it might not strictly be an oil in the normal meaning of the word. Two types of emulsions can be identified, oil-in water (o/w) and water-in-oil (w/o) emulsions, depending on which phase, the water or the oil, is the continuous or discontinuous phase. Examples of o/w emulsions include paints, glues, milk, bitumen emulsions, mayonnaise, agrochemical formulation. Liquid margarines are instead a common example of a w/o emulsions.

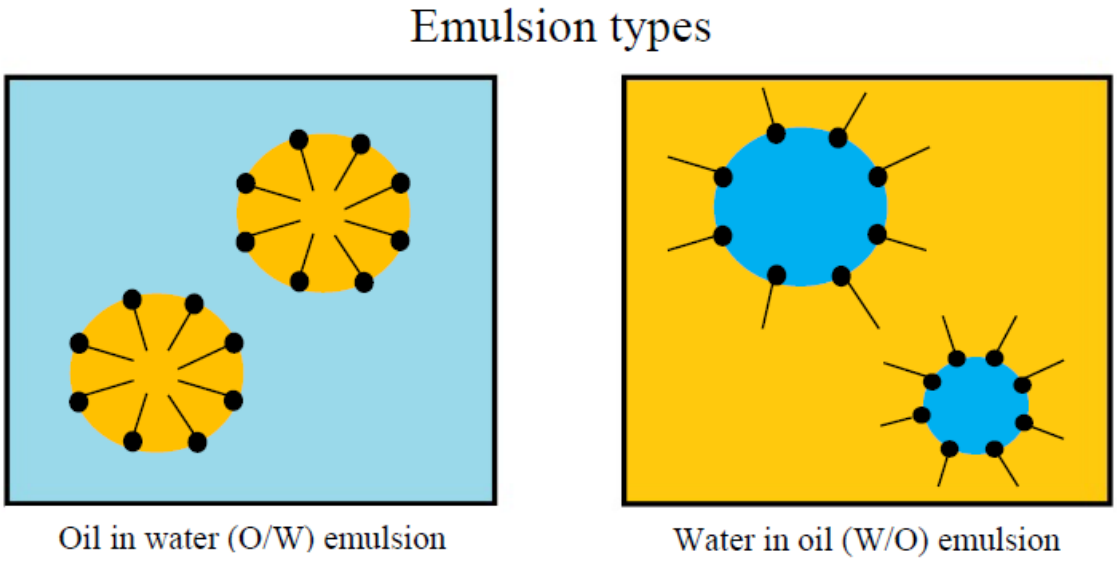

Figure 5. Schematic of o/w and w/o emulsions [14].

Emulsions can also be divided into different categories based on their droplet size. Microemulsions, whose name is misleading, have a droplet diameter in the range 10-100 nm, meanwhile nano-emulsions lay in the interval 40-200 nm [15]. Above this value they are simply called macroemulsions. As it can be noted, the ranges overlap and unfortunately

change in the literature. Besides the mean droplet diameter, the main difference between the two systems is that microemulsions are a mono-phase and thermodynamically stable system, which is formed spontaneously and can have different properties than nano-emulsions and normal emulsions, such as optical clarity. Emulsions (macro and nano) are thermodynamically unstable systems and therefore have a tendency to phase separate (i.e. form two separate oil and aqueous phases) over time.

In order to be stable over time an emulsion must be composed of, together with water and oil, at least one other component: an emulsifier. The most common type of emulsifier for the formulation of emulsions are small molecular weight surfactants as amphiphilic polymers. The amphiphilic character of surfactants enables them to link the two phases together, by having the hydrophobic part directed towards the oil phase and the hydrophilic part directed towards the water phase. This forms an elastic film at the interface that prevents the discontinuous phase droplets to coalesce. Emulsifiers also help to finely disperse either oil into water or water into oil by reducing the interfacial tension. As a consequence, two characteristics are of primary importance when choosing emulsifier: firstly, it needs to be sufficiently surface active to decrease the oil-water interfacial tension to low values and secondly it must diffuse quickly to the newly created interface when water and oil are mixed.

When the emulsification technique and process parameters are fixed, a lower interfacial tension plays an important role in favouring the creation of small droplets. Looking at *Figure 6* it could be said, in other words, that it is one of the main factors of the break-up process in the high shear zone. On the other hand, the fast diffusion of the emulsifier to the newly created droplet is crucial, as, only if the new interface is rapidly covered by an emulsifier monolayer, will that interface be stable against coalescence and avoid an increase in droplet size. Both characteristics are therefore necessary to obtain good stability in an emulsion.

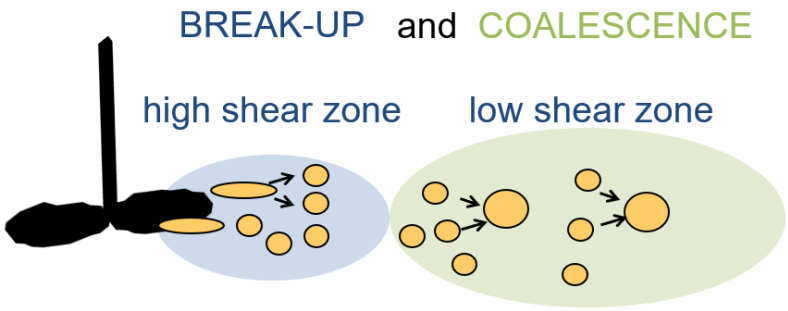

Figure 6. Scheme of emulsification process. Droplet break-up occurring close to the propeller in the high shear zone and immediate coalescence occurring in the low shear zone.

3.3.1. Stability

An emulsion is stable when the droplets retain their initial character and remain uniformly distributed throughout the continuous phase. Emulsions can undergo six different types of instability processes, namely: sedimentation, creaming, flocculation, coalesce, Ostwald ripening and phase inversion. All of these destabilization mechanisms, shown in *Figure 7*, will be briefly introduced next.

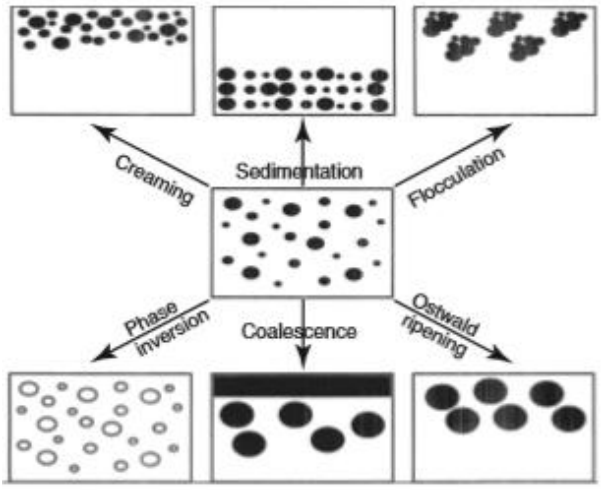

Figure 7. Schematic representation of the various breakdown processes in emulsions [16].

3.3.1.1. Sedimentation and Creaming

In sedimentation, the emulsion droplets tend to sink down and gather at the bottom of the container, meanwhile in creaming they go up and float at the top. The driving force in this process is the difference in density between the continuous and discontinuous phases of the emulsion, as it can be seen in Stokes equation (*Equation 1*) for the settling velocity (v).

$$v = \frac{g}{18\ \mu_{cont.ph.}} * \left(\rho_{drop} - \rho_{cont.ph.}\right) * D_{drop}^{2}$$

Equation 1. Stokes law for settling velocity [16].

Furthermore, *Equation 1* shows that these destabilizing processes are directly proportional to the droplet size of the emulsion and inversely proportional to the viscosity of the continuous phase. Therefore, smaller droplets and more viscous continuous phase can provide better stability against both sedimentation and creaming.

3.3.1.2. Flocculation

Flocculation occurs when there is not enough repulsion between the droplets and van der Waals attractive force prevail. The droplet size does not change, as the droplets simply aggregate into clusters, maintaining the same surface area. Since no phase separation occurs, the phenomenon can be easily reversed by shaking [16].

3.3.1.3. Coalescence

Coalescence is an irreversible phenomenon where emulsion droplets merge upon colliding, thus forming larger droplets. Ultimately, this process can lead to phase separation. It all starts with the creation of a bridge between the two colliding droplets. The bridge is unstable and grows during the process. In stable emulsions the emulsifier prevents bridge formation. Therefore, choosing the emulsifier with the right molecular properties is essential to have good stability against coalescence.

3.3.1.4. Ostwald ripening

Ostwald ripening is an instability phenomenon arising from the fact that smaller droplets have a higher internal pressure than bigger particles. This pressure gradient, drives the molecular diffusion of the liquid contained in the drops from the small to the big drops, causing the growth of the big drops at the expense of the small ones. Eventually, this leads to complete separation of the phases. For emulsions with drops in the micrometer size range, this phenomenon is normally quite slow and can last for years.

3.3.1.5. Phase inversion

This refers to an exchange between the dispersed phase and the continuous phase. For example, an o/w emulsion may with time or a change of conditions, such as concentration, temperature and pressure, invert to a w/o emulsion. Dramatic changes in properties of the emulsions, including viscosity and drop size could occur. However, phase inversion does not necessary lead to destabilization of the emulsion.

3.3.2. Emulsification techniques

The section 3.3.1.1 discussed how decreasing the mean droplet diameter of an emulsion increases stability against creaming or sedimentation. Furthermore, if the drop size distribution is narrow, Ostwald ripening can be reduced, as droplets with the same size would have the same pressure. To obtain these properties the emulsification technique plays an important role. The four most common emulsification techniques are briefly presented here, together with their advantages and disadvantages.

3.3.2.1. Mechanical mixing devices

Mechanical mixing can be performed by a wide range of propellers and turbines with diverse geometries. One of the most common devices is the rotor-stator mixer, which is composed of a rotor that rotates at high speed in a stationary stator. If the viscosity is too high the rotor-stator designs are often combined with anchor or turbine agitators to improve mixing. Complex intermeshing rows of concentric teeth together with a higher mixing speed can be chosen to increase the disruptive force of the instrument and create finer droplets. However, droplet diameters in the sub-micrometer range are difficult to achieve and therefore these devices can be successfully used, such as in this project, as pre-emulsification techniques, i.e. lower energy input devices are used first, followed by higher energy mixing.

3.3.2.2. High pressure devices

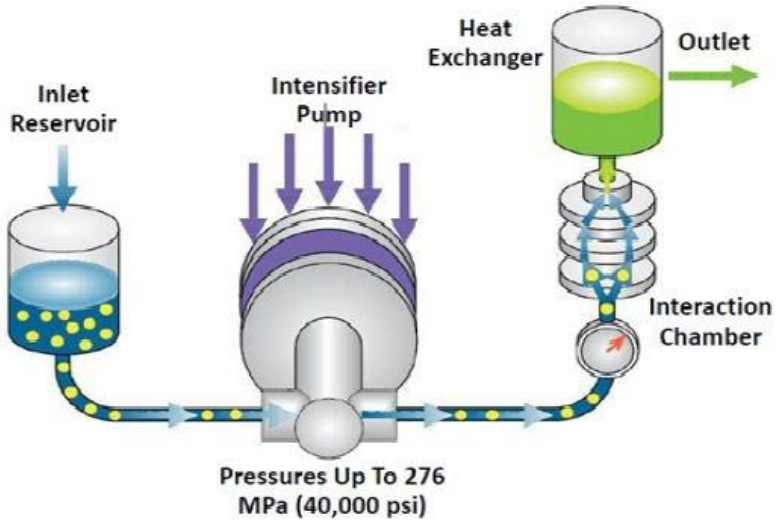

Figure 8. Microfluidizer set-up [18].

Homogenizer are high-pressure devices that are suitable for the formulation of sub-micron emulsions. The size of the single droplets depends on the balance between two opposing mechanisms: droplet disruption and droplet coalescence. The smallest droplet diameter achievable by these instruments varies based on homogenizer design and operating conditions, sample composition and the physico-chemical properties of the component phases [19]. Two types of devices belong to this category: (1) high pressure valve homogenizers and (2) microfluidizers.

In *Figure 8*, a scheme of a typical microfluidizer is shown. Once the pre-emulsion is prepared and introduced in the inlet reservoir it goes through an intensifier pump that can reach pressures of up to 2500 bar. Then, the flow is divided and reunited in the interaction chamber where high energy collisions are generated. Different geometries of the interaction chamber are designed for different types of emulsions. Due to the concentrated heat produced in the chamber, an ice bath or an automatic refrigerating system can be installed to control the temperature. In this system, droplet size tends to decrease with increasing homogenization pressure, number of passes, emulsifier concentration and decreasing disperse-to-continuous phase viscosity ratio. Usually there is a linear relationship between the logarithm of the droplet diameter and the logarithm of the pressure [20]. Finally, the instrument is robust and easily used on industrial scale and is capable of producing more monodisperse emulsions than mechanical mixing and ultrasonic devices.

3.3.2.3. Ultrasonic devices

Ultrasonic devices are the other high-energy alternative for the formulation of sub-micron emulsions. Contrary to microfluidizers, the energy is provided to the system via high intensity ultrasonic waves, through a metallic probe. The propagation of these waves in the emulsion generates a cycle of high and low pressure leading to cavitation. This breaks the oil droplets in fine drops and can sometimes induce chemical changes that can enhance emulsion stability.

The droplet size is influenced by the intensity of the ultrasonic waves and the residence time, higher intensity and longer times results in finer droplets. Both batch and continuous systems exist, but the high-energy consumption makes it less appealing for industrial scale. Concentrated heat can also cause problems if working with heat-sensitive compounds.

3.3.2.4. Membrane emulsification

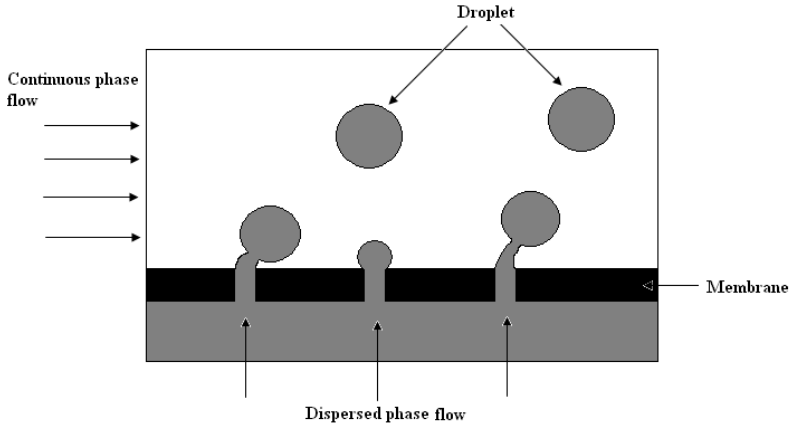

Figure 9. Schematic of crossflow membrane emulsification [21].

The working principle of membrane emulsification is quite simple and is shownin *Figure 9*. Due to a pressure difference between two compartments of a porous membrane the dispersed phase is forced through the pores into the continuous phase containing the emulsifier. The droplets are formed one by one in what is called the drop-by-drop mechanism and dragged away by the continuous phase in a crossflow mode (batch or continuous), as depicted in *Figure 9*, or in a stirred cell (batch). Membrane emulsification provides good control over emulsion microstructure, generating the most monodispersed systems compare to all other techniques. The much lower energy input required for processing could make it a suitable option for low-cost applications. Furthermore, the lower level of shear and thermal stress would allow membrane emulsification to be used for sensitive materials, like micronutrients and proteins. Unfortunately, the major drawback of this system is the low dispersed phase flux, that together with the high membrane costs could make this technique economically interesting only for the production of high value products, for example, luxury cosmetics, drug delivery systems and chromatography beads [21].

3.4. Pickering emulsions

Pickering emulsions are emulsions stabilised by solid particles. Although the discovery that small solid particles could provide good stabilization toemulsions was made over a century ago concurrently but independently by Pickering [22] and Ramsden [23], recently there has been growing interest in this field. In certain industrial applications such as food, cosmetics and pharmaceuticals there is a strong need for a biocompatible, biodegradable and sustainable alternative to petroleum-based surfactants. Furthermore, some common surfactants have been shown to be harmful for the environment, especially for aquatic organisms [24], as well as potential skin irritants (in humans and animals) as demonstrated with some conventional surfactants used in topical formulations [25].

Pickering emulsions stabilized by different inorganic and organic particles, such as silica and clay (inorganic) and starch, cellulose and chitin (organic) have been studied. Pickering particles from cellulosic materials, in particular, such as nanocellulose could help solve the drawbacks of conventional surfactants, thanks to their biocompatible and biodegradable characteristics. These nano- or micro-particles have been reported to strongly adsorb at the oil-water interface in a form of a densely packed layer that protects the droplets against flocculation and coalescence via a steric and/or electrostatic mechanism [26].

There is also evidence that, in some systems, weak flocculation of the particles improves emulsion stability by creating a three-dimensional network in the aqueous phase that keep the droplets separate from each other [28]. The stabilization properties of different particles, however, strongly depends on several factors such as the size, shape, surface energy and charge, and wettability [29]. The latter is an essential characteristic of the particle in order to be a good emulsifier.

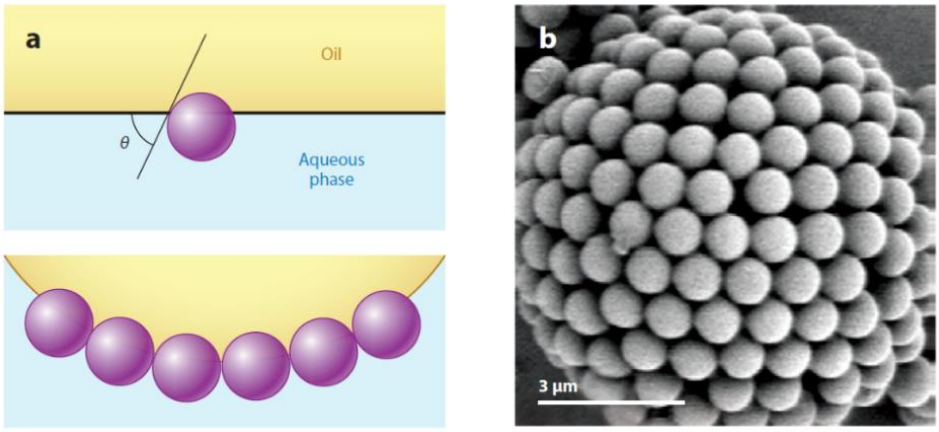

Figure 10. (a) Position of a small spherical particle at a planar oil-water interface for a contact angle less than 90◦, and corresponding positioning of particles at a curved interface of an oil-in-water emulsion. (b) Polystyrene particles assembled on an oil droplet in water [27].

Particles suitable for Pickering stabilization of emulsions should be partly wetted by both oil and water. A measure of the affinity of the particle to one of the two phases is the contact angle θ. As it can be seen in *Figure 10*, if the water contact angle is in between 0° and 90° the particle will be mostly surrounded by water (hydrophilic particle), if the water contact angle is from 90° till 180° then they will have more affinity with the oil (hydrophobic particle). In emulsions with the same volume for both phases, hydrophilic particles will tend to form o/w emulsions, meanwhile hydrophobic particles will most likely form w/o emulsions; this is called Bancroft's rule [27]. Finally, the size of the particle is also an important parameter if emulsions with small droplets are required, it is necessary that the dimensions of the emulsifying particles don't exceed the dimensions of the desired droplet size.

3.5. Pickering emulsions as topical delivery systems

The applications of Pickering emulsions in the delivery of drugs through skin relies on their specific properties manifested in vivo and the development of new dosage forms that show improved emulsion properties [30]. The main in vivo benefits of Pickering emulsions are the lower skin irritancy compared to small molecule surfactant-based creams and the specific

interactions with biological interfaces. Emulsion properties such as enhanced stability, due to steric and/or electrostatic repulsion, and viscosifying of the cream without the use of polymeric thickeners are further advantages of this type of formulation that could be beneficial in the cosmetic/ pharmaceutical sector.

Droplet size and viscosity are two fundamental parameters in regulating the release of an active principle from an emulsion to the skin. In general, not only for Pickering emulsions, smaller droplet size means higher surface to volume ratio and therefore faster adsorption of the drug to the skin. The presence of a densely packed interfacial layer around the droplet could also affect the release profile of an active by acting as a controlling barrier to produce a sustained drug release [30].

To better understand the mechanism of skin adsorption of a drug from o/w Pickering emulsions, Frelichowska et al. [31] provided a general overview of all possible transport phenomena that could take place and explained which ones are likely to be predominant. The first step is the drug release from the emulsion droplets in the continuous phase, this release behaviour is commonly referred to as bioavailability. Since the dense coating of the solid particles around the droplets act as a barrier to the diffusion of the active in the surrounding medium, the result is an encapsulation system that delays drug release. Accordingly, the permeation of the drug through the skin should be slower. In reality, at least for hydrophilic actives that do not get trapped in the stratum corneum (*Figure 11*), the drug flux is measured to be faster than conventional surfactant-based emulsions [31]. This has been explained by the dominant effect of direct drug transfer from the emulsion droplets to the skin surface. The adhesion energy of Pickering emulsions droplets to the skin has been measured to be higher than that of surfactant-coated drops, which means longer contact time of the droplets with the skin and consequently faster drug release.

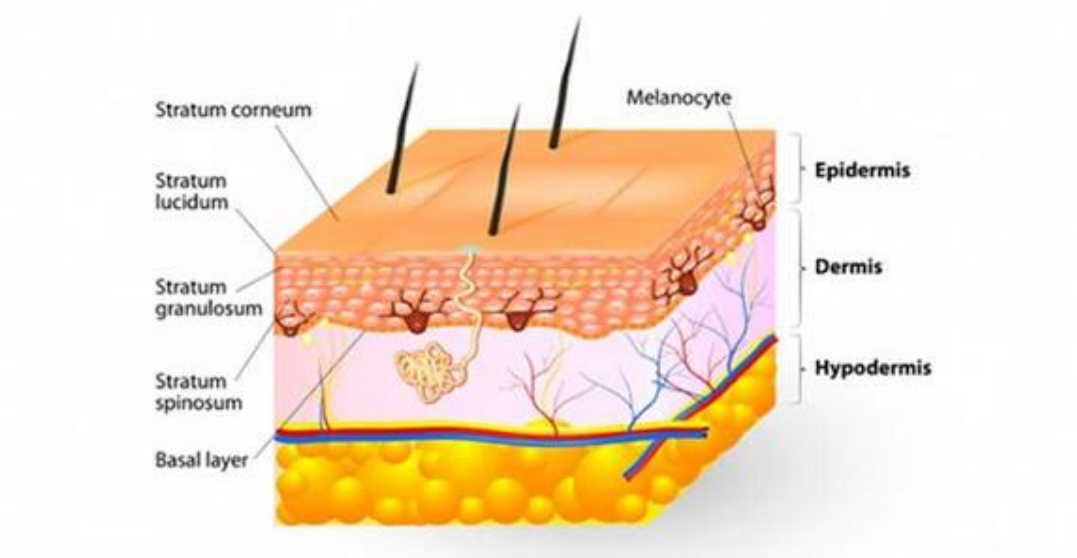

Figure 11. Scheme of the different layers of the skin [32].

Finally, particle-stabilized droplets are not believed to be able to penetrate the skin, because of their dimensions (100-300 nm) [31]. On the contrary, the penetration in the first layers of the skin of some of the small particles (6-10 nm) with adsorbed active has been observed and could give a possible supplementary transport mechanism for the drug [31].

3.6. Lutein

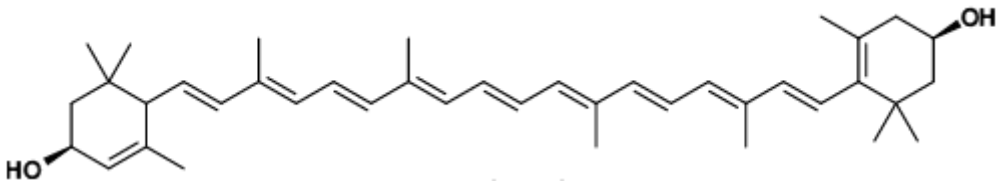

Figure 12. Chemical structure of Lutein.

Carotenoids are a group of polyenes consisting of 3-13 conjugated double bonds that may have six-carbon ring structures at one or both ends. Depending on whether they contain oxygen or not, they are classified as xanthophylls or carotenes respectively [33]. Lutein, together with zeaxanthin, belongs to the family of the xanthophylls, due to the presence of the hydroxyl groups at both ends (*Figure 12*).

The exposure of unprotected human skin to sun ultraviolet radiation can generate in an inflammatory reaction. This is caused by different possible mechanisms, such as the formation of reactive oxygen species and free radicals that can also induce skin aging, carcinogenesis and erythema [45]. Lutein is an antioxidant that is naturally present in the human skin, taken in from a varied diet, but lutein is not synthetized by the organism. Lutein supplements taken either orally or topically can help in enhancing its concentration in the skin. Due to its strong antioxidant properties, oral and topical administration of this active has been shown to efficiently reduce skin lipid peroxidation and to develop a photo-protective activity against UV light irradiation [34, 35].

All carotenoids in their natural environment are stable against degradation. However, in their isolated form they are prone to chemical degradation induced by light, oxygen, pH and temperature differences. Therefore, when used in formulations, all these parameters need to be controlled.

4. State of the art

4.1. Pickering emulsions stabilized by CNCs

Among the many different particles that have been investigated for formulation of Pickering emulsions, CNCs have attracted the interest of both academic researchers and industrial product developers due to the technical properties and environmentally friendly profile described in detailed in the previous sections of this report. Several studies have described the performance of CNCs in connection to their potential uses in latexes, food and pharmaceutical formulations [36][29][13].

The work cited in this section is only a small part of the published literature on Pickering emulsions stabilised by CNCs but includes the studies that are most crucial to understand the goals and results of this project. CNCs have been tested as emulsion stabilizing agents on their own as well as in combination with co-stabilizer, such as adsorbing water-soluble cellulose derivatives or surfactants. An overview of the relevant and recent literature on CNC stabilized emulsions and their main conclusions are presented in *Table 1*.

Table 3. Summary of relevant literature on CNC-stabilised Pickering emulsions.

Stabilizing agents	System	Emulsification technique	Main findings	Ref.
Cellulose Nanocrystals (CNC)	10 wt.% oil 0.05-2 wt.% CNC CNC/Oil = 0.45-18/100	Microfluidization 600-1300 bar	Decreasing droplet size with increasing pressure Decreasing droplet size with increasing CNC conc. Minimum at 1200 bar, 0.75 wt.% CNC (d= 0.92 µm)	[29]
Didecyldimethylammonium Bromide (DMAB) or Cetyltrimethylammonium Bromide (CTAB)	50 wt.% dodecane oil 0.50 wt.% CNC CNC/Oil = 0.5/100	Sonication	Adsorbed surfactants change the CNCs wettability Higher surfactant concentration lower droplet size and higher stability (d from ca. 40 to 8 µm)	[38]
CNC + Methyl Cellulose (MC) or Hydroxyethyl Cellulose (HEC) or Dextran (DEX)	50 wt.% dodecane oil 0.1-1 wt.% CNC CNC/Oil = 0.1-1/100 CNC/Polymer = 1/0.67	Sonication	Synergistic effect of CNC + Polymer No synergistic effect for CNC + DEX CNC/Polymer = 1/0.05 (min. to saturate CNCs surface) Decreasing droplet size with increasing CNC conc. Minimum at 1 wt.% CNC (d= 2.8 µm for HEC and 1.9 µm for MC)	[39]
CNC + Methyl Cellulose (MC) or Hydroxyethyl Cellulose (HEC) + Tannic Acid (TA)	25 wt.% corn oil 0.25 wt.% CNC CNC/Oil = 1/100 CNC/MC = 1/1	Sonication	Complexation of the drops shell with Tannic acid Redespersable emulsion with same droplet size Importance of mixing order	[41]

Bai et al. [29] was the first to report the production of stable Pickering emulsions with CNCs as the only stabiliser using microfluidization. In their work stability and mean drop diameter in o/w emulsions containing 10 wt.% oil were investigated at different CNC concentrations (0.05-2 wt.% of the aqueous phase) and different microfluidization pressures (600-1300 bar). The minimum attainable drop size was found to depend on both the microfluidization

pressure and the CNC concentration. At a constant microfluidization pressure of 900 bar, the average emulsion droplet size decreased down to values under 1 μm as the CNC concentration increased up to 0.75 wt.% and remained constant as the CNC concentration was further increased up to 2 wt.%. Increasing the CNC concentration also favoured stability against creaming, both due to the reduction in drop size and to a slight increase in the aqueous phase viscosity. Higher microfluidization pressures favoured a reduction in drop diameter to a minimum value of 920 nm at around 1200 bar. An unexpected increase in droplet size occurred at 1300 bar. Finally, different food-grade oils were tested and all of them seemed to be able to form stable emulsions with CNCs, including the medium chain triglycerides (MCT) used in this project. These systems remained stable against drop coalescence over a wide range of pH (3-10), NaCl concentrations (≤ 100 mM) and temperatures (30-90 °C) [29]. Their work is the first example showing the possibility of producing submicron CNC-stabilized emulsion droplets by using a high-energy emulsification technique, such as microfluidization [29], instead of the probe sonication used in previous work [40]. However, relatively large amounts of CNCs (0.75-2%) was needed to stabilize an emulsion composed by only 10% of oil, which in terms of CNC/oil ratio means that 18 g of CNCs are needed for every 100 g of oil.

In the second of a series of two papers, Bai et al. [37] reported on the ability of these CNC-stabilised emulsions to modulate the release of emulsified lipids in the human digestion system. By monitoring the free fatty acids (FFA) in a small intestine stage of a 3-stage gastrointestinal tract model, it was shown that the final extent of FFAs released was reduced by 40 %. Based on this finding, these systems are considered to offer very interesting possibilities for the development of emulsified functional foods [37].

The project presented here builds on work by Hu et al. [38] showing that the ability for CNCs to stabilise emulsions can be improved by adding adsorbing co-stabilisers. For example, anionic CNCs could be surface modified *in situ* to tailor emulsions by adding cationic alkyl ammonium surfactants such us DMAB and CTAB [38]. Both surfactants adsorb onto CNCs and change the particle wettability based on their concentration. At low surfactant concentrations, individual surfactant molecules adsorb with the alkyl tails pointing out from the CNCs rendering hydrophobic particles. At higher concentrations, above the surfactant critical micelle concentration, the surfactants adsorbed on the CNC surface in the form of aggregates, resulting in a lower hydrophobicity of the particles. Adding surfactants, changed CNC wettability resulting in enhanced o/w emulsions stability and a decreased droplet size from ca. 40 μm to 8 μm. Increasing the amount of DMAB, which is the more hydrophobic surfactant, showed also the possibility of stabilizing w/o emulsions. Finally, the surface modification of the CNCs is shown to be a useful technique to sensibly increase the stabilizing properties of the CNCs [38]. Compared to Bai et al. [29], the stability of a 50%-oil emulsion is reached with only 0.5% of CNCs, which in terms of CNC/oil ratio is equal to 0.5 g for each 100 g of oil, 36 times less than using CNCs alone.

Other, more biocompatible/food grade, surface active compounds have also been used to surface modify CNCs for Pickering emulsions. Cellulose derivatives such as hydroxyethyl cellulose (HEC) or methyl cellulose (MC) have been investigated by Hu et al. to show the effect of water-soluble polymers on the properties of Pickering emulsions stabilized by CNCs [39]. Emulsions with only CNCs, with CNCs+MC, with CNCs+HEC, or with just HEC or

MC were analysed. Both polymers were shown to adsorb on the CNC surface to reach full coverage of CNCs at CNC/HEC or CNC/MC ratio of 1/0.05 in mass. Emulsions stabilised by both CNCs+HEC and CNCs+MC, displayed higher stability to coalescence and smaller droplet sizes than emulsions stabilised by CNCs, HEC and MC alone, proving the synergistic effect of adsorbing surface-active polymers to the CNCs. In their study, smaller emulsion droplet sizes were obtained by increasing the CNC concentration, at a constant CNC to polymer ratio of 1/0.067. By calculating the surface coverage of the oil droplets to maintain stability, only 20% was seen to be covered by surface modified CNCs, with the remaining 80% stabilized by HEC or MC alone. This value is considerably lower than the 84% found by Capron et al. [40] necessary to obtain stable droplets with unmodified (and desulfated) CNCs, which further confirms that with surface modified CNCs lower amounts of material are needed to stabilize the same amount of oil. However, in Hu et al. [38] work the size of the emulsion droplets was still above one micrometer, no variation of the CNC/Polymer ratio was studied and the stability of the samples was followed for only 6 days.

The properties of the CNC+HEC and CNC+MC emulsions was further investigated bythe same research group; n their 2016 work, Hu et al. [41] reported on the addition of tannic acid to the CNC+HEC or CNC+MC-stabilised emulsions and how it made it possible to freeze-dry and re-disperse the emulsions after drying, maintaining the same droplet size throughout the process, therefore substantially preventing droplet coalescence under a variety of harsh conditions. Tannic acid was shown to form colloidal complexes with the HEC and MC polymers strengthening the CNC-polymer shell around the oil droplets which enhances stability during freeze-drying, prevents coalescence after drying, and allows for redispersion. The order of mixing the different compounds was also found to play an important rule, as only the sample in which MC or HEC was mixed with CNCs before emulsification with tannic acid could be successfully freeze-dried and re-dispersed [41].

4.2. Pickering emulsions as topical drug delivery systems

The effect of Pickering emulsions as topical drug delivery systems will differ depending on which type of active substances are to be delivered. In the literature, topical delivery of hydrophilic drugs in w/o emulsions and lipophilic ones in o/w emulsions have been investigated [31] [41] (*Table 2).* To the best of our knowledge there are no previous reports of using CNC-stabilized emulsions as carrier formulations for drug delivery.

Table 4. Summary of the relevant literature on Pickering emulsions in drug delivery.

Stabilizing agents	System	Active	Main findings	Ref.
Hydrophobic silica particles	w/o emulsion 50 wt.% oil 1 wt.% silica particles 0.9% caffeine Franz cells membrane: pig skin Receptor fluid: 0.9% NaCl	Caffeine (hydrophilic)	Enhanced skin absorption, compared to classical surfactant-based emulsion, due to specific interactions of the formulation with the skin structures, higher adhesion energy between them, and penetration of nanoparticles in the first layers of the stratum corneum.	[31]
Hydrophobic silica particles	o/w emulsion 10 wt.% oil 7 wt.% silica particles 0.1% retinol Franz cells membrane: pig skin Receptor fluid: 1.5%	All-trans retinol (highly hydrophobic)	No skin permeation due to high affinity of the molecule with the lipidic medium of the stratum corneum. Retinol strongly retained in the stratum corneum and reached the viable epidermis and dermis to a lesser extent in comparison to the surfactant-based emulsion.	[41]

	Brij®98 and 0.5% alpha-tocopherol acetate			
Starch	o/w emulsion 56 wt.% oil 200 mg Starch/ml oil Flow-through diffusion cells Membrane: pig skin Receptor fluid: 0.1 M phosphate buffer	Methyl salicylate (hydrophobic)	Accelerated penetration to the dermis and permeation through all the skin compared to standard solutions of methyl salicylate	[42]

Frelichowska et al. [31] reported on the in vitro skin permeation of caffeine in w/o silica-stabilized emulsions compared to conventional surfactant-stabilised emulsions via Franz cell set-up with excised pig skin. Both emulsions presented the same composition, droplet size, volume fraction of dispersed phase, viscosity, differing only by the nature of the stabilizing agent, i.e. different oil/water interfacial layer properties. The results showed a caffeine pseudo-steady state flux of 25 μg cm^{-2} h^{-1} for the Pickering emulsion, three times higher than classical emulsions (9.7 μg cm^{-2} h^{-1}). Caffeine permeated well through the skin because of low Mw and low log P (partition coefficient). The enhanced skin absorption of caffeine from Pickering emulsion was suggested to be due to specific interactions of the formulation with the skin structures, due to a higher adhesion energy between them, and to the penetration of nanoparticles in the first layers of the stratum corneum, confirmed by SEM observations revealing the presence of silica aggregates till half of the stratum corneum depth (5 μm). Adsorption of caffeine to those silica aggregates was suggested to provide a supplementary transport mechanism for the drug.

In the same way as the caffeine in w/o emulsions, all-trans retinol in o/w emulsions have been compared to a classical surfactant-based emulsion in *in vitro* skin penetration tests (Franz cell with pig skin) [41]. In this case, the amount of retinol getting through the skin was negligible for both formulations, due to the high affinity of this high hydrophobic molecule with the lipidic medium of the stratum corneum. Furthermore, the amount of retinol found in the skin was also identical for both systems. What was different was, in the first place, that the retinol from Pickering emulsion was strongly retained in the stratum corneum and reached the viable epidermis and dermis to a lesser extent in comparison to the surfactant-based emulsion. Secondly, the retinol stored inside the stratum corneum during 24 h exposure was then also slowly released for a supplementary 24 h delay without exposure.

Finally, the considerations made for retinol cannot be generalized for all lipophilic compounds as it has been shown that less hydrophobic drugs loaded in o/w Pickering emulsions showed, on the contrary, accelerated penetration to the dermis and permeation through all the skin [42]. Although, the mechanism of how drugs in Pickering emulsions adsorb and diffuse through the skin is not clear yet, it is clearly established that Pickering emulsions do not behave like simple penetration enhancers that increase the permeability of the stratum corneum by fluidization of the intercellular lipids.

5. Materials and methods

5.1. Materials

All materials used in this project are described in *Table 3*.

Table 5. List of materials used.

Substance	Trade Name/Product	Description	Supplier
Oil	Miglyol 812 N	Mixture of Decanoyl- and Octanoyl Glycerides (Fatty Acid Esters)	IOI Oleochemical
Particle	NCC^{MC} CelluForce	Cellulose Nanocrystals	CelluForce
Polymer	METHOCELL^{TM} K4M	Hydroxypropyl Methylcellulose	Dow Chemical
Carotenoid	FloraGlo Lutein 20% SAF	Suspension of 20% Lutein in Safflower Oil	DSM Nutritional Products
Carotenoid	Lutein Analytical Standard	Isolated Lutein	Sigma-Aldrich
Surfactant	Lipoid S100	Soybean Lecithin, non-ionic surfactant	Lipoid GmbH
Surfactant	Tween 80	Polysorbate 80, non-ionic surfactant	Sigma-Aldrich

The NCC^{MC} CelluForce are spray-dried cellulose nanocrystals produced by sulfuric acid hydrolysis of bleached soft wood kraft pulp and sold in the Na^+-neutralized form. The most relevant characteristics of the HPMC used in this project are shown in *Table 4*.

Table 6. Characteristics of the HPMC.

Property	Value
Particle Size	R_h = 42 nm PI = 0.4
Viscosity	η = 4000 mPa s
Molecular Weight	317 kg/mol
Methoxy Content	21.5%
Hydroxypropoxy Content	9.5%

All water used was ultrapure water (Type 1) with a conductivity of 18.2 MΩ cm^{-1}. The Ethanol used in the drug delivery study had a purity of 99.5%.

5.2. Dispersion of cellulose nanocrystals

Three different stock dispersions of 1 L with a CNC concentration of 2 wt.% were prepared by mixing 20 g of CNC powder with 1 L of water under mechanical stirring. The dispersion was then sonicated in an ice bath using a probe sonicator Vibracell VCX-750 (Sonics & Materials) in order to break down the remaining agglomerates into individual crystals (*Figure 13*).

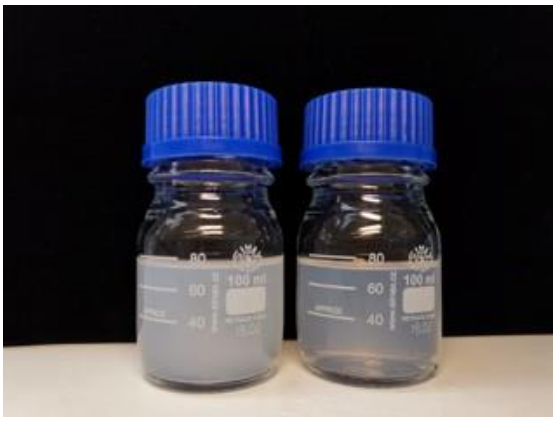

Figure 13. From the left: non-sonicated CNC stock dispersion, sonicated CNC stock dispersion.

The amplitude was set to 80 % and the time was chosen in order to provide 5000 J/g of energy to the system. To get rid of possible residuals from the sonicator probe, the dispersion was filtered through Whatman 541 (GE Healthcare) filter papers and then stored in refrigerator for the duration of the project. The concentration was finally checked by thermogravimetric analysis.

5.3. Characterization of cellulose nanocrystals

CNCs were characterized in terms of particle size, zeta potential and surface charge density. Particle size was measured by dynamic light scattering (DLS) using a Nanosizer ZS (Malvern Instruments). The samples were diluted to a concentration of 0.025 wt.% with a solution of 10 mM NaCl in order to reduce multiple scattering and limit particle-particle interactions. The measurement was performed in triplicate.

In order to have a precise measure of the length and height of the CNCs, the stock suspension diluted to 0.002 wt.% and imaged by Atomic Force Microscopy (AFM) using PeakForce tapping on a Multimode AFM Nanoscope V (Bruker). A volume of 25 µL of PAH (polyallylamine hydrochloride) solution (0.1 wt.%) was spin-coated onto a piece of freshly cleaved mica. The CNCs sample were then spin coated onto the substrate at 3500 rpm for 1 min.

The AFM image was used to calculate the length and height ranges of the CNCs. The calculation was performed using NanoScope Analysis 1.8 (Bruker) software and the final values are the result of 100 measurements.

Zeta potential was measured through electrophoretic light scattering by a Zetasizer (Malvern Instruments, UK). The samples were diluted to a concentration of 0.1 wt.% with a 10 mM NaCl solution to reduce the double layer thickness around the CNCs. The final value is the result of three replicates.

The surface charge density of the CNCs was measured by conductometric titration. The sample was passed through an ion-exchange resin Dowex Marathon^{TM} C (Sigma-Aldrich) in order to convert the CNCs from neutral to acid form. The acidic CNCs were titrated with a 2 mM NaOH solution following the change of conductivity over the entire time.

5.4. Dissolution of hydroxypropyl methylcellulose

The direct addition of HPMC to water results in the formation of lumps due to the incomplete wetting of the powder. The avoid that, the hot water method was used to dissolve the polymer in water. Two batches of 1 L were prepared. Half the total amount of water was warmed up to 75 °C. The powder was continuously poured, while stirring, until it was completely dissolved. The remaining water was added as cold water and the dispersion was stirred in an ice bath until it became transparent. Finally, the solution was characterized in terms of concentration, via thermogravimetric analysis, and in terms of polymer size, via DLS.

The stock solutions were stored in refrigeration for the whole duration of the project.

5.5. Preparation of emulsions

All formulations prepared in this project contained 50 wt.% oil, unless otherwise stated in the text. For each experiment an emulsion of 150 g was produced. The amount of CNC in the system was varied from 0.03 to 0.75 wt.% of the aqueous phase. Meanwhile the CNC/HPMC (wt./wt.) ratio was changed from 1/0.05 to 1/1, which means that the amount of HPMC ranged from 0.005 to 0.75 wt.% of the aqueous phase. The emulsion produced for the Franz cells experiments contained 0.025% or 0.5% lutein. FloraGlo was diluted with Miglyol to a concentration of 0.05% or 1% of lutein and was sonicated in an ultrasound bath for 10 min. The oil solution was then probe sonicated using a Vibracell VCX-750 (Sonics & Materials) in an ice bath for 3 minutes with a 3 s pulses and 3 s pause. Finally, the oil phase with or without lutein was emulsified with the aqueous phase.

The emulsification protocol is illustrated in *Figure 14*.

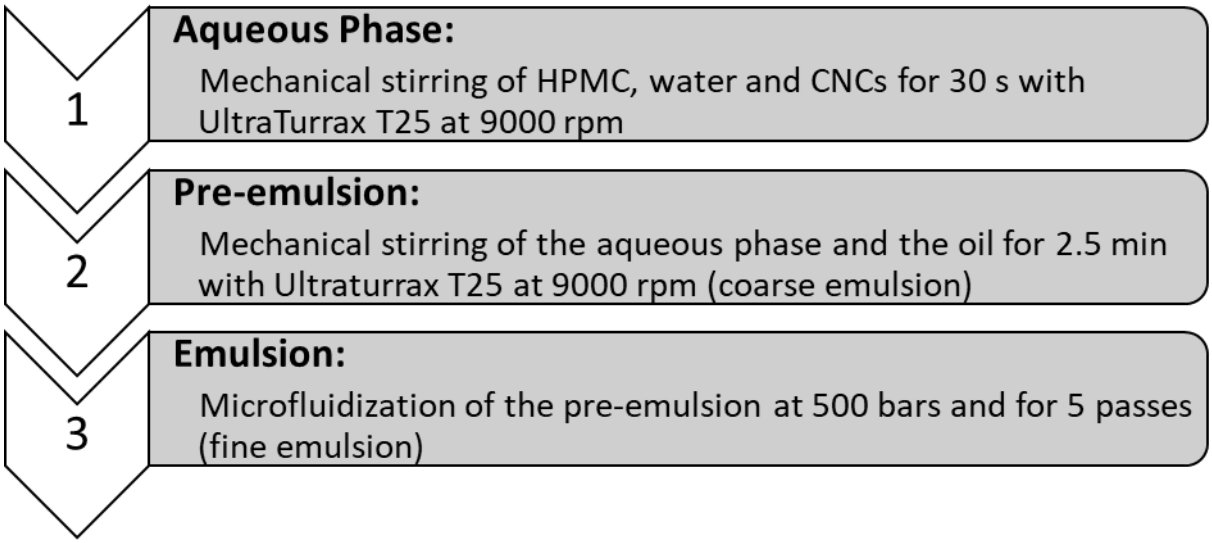

Figure 14. Emulsification protocol**.**

The pressure and number of passes in the microfluidizer were kept constant during the project, unless otherwise stated. The interaction chamber was a H10Z (Microfluidics) with 100 μm microchannels.

The Lipoid emulsion contained 2.5% of Lipoid in the aqueous phase. The same protocol of *Figure 14* was followed, but with a pressure of the microfluidizer of 200 bar. The aqueous

phase was prepared by dissolving the Lipoid in water at 70 °C and stirred in the UltraTurrax T25 (IKA) at 9000 rpm for 30 s.

5.6. Design of experiments

The software MODDE Pro v. 12.1 (Umetrics) was used to determine a suitable set of experiments that could give most of the information with the least number of experiments. Both the CNC concentration and the CNC/HPMC ratio were defined in the programme as "quantitative factors" since the amount of one was not dependent on the amount of the other and so it was not considered necessary to define these as "mixture (fraction) factors". The most appropriate design that provided the best fit of the whole study range appeared to be a central composite orthogonal (CCO) design, which can be seen in *Figure 15*.

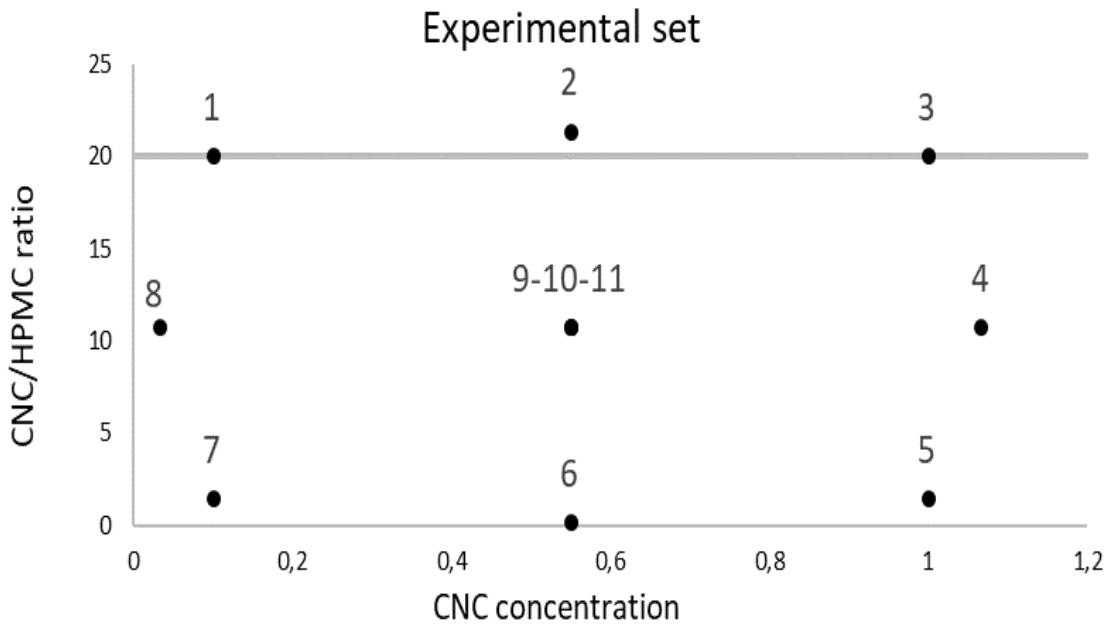

Figure 15. MODDE original experimental set (CNC/HPMC = 1/0.05 is the value under which CNC surface is completely saturated with HPMC).

This design includes 7 experiments (1-3-5-7-9-10-11) that are contained in the study range, which are called "full factorial points" and 4 experiments (2-4-6-8) that are outside the study range, that are called "star points". Points 9-10-11 are triplicates that are needed by the software to assess the reproducibility of the system.

Experiments 5 and 6 showed a too high viscosity due to the presence of both HPMC and CNCs in high concentrations. This resulted in the clogging of the microfluidizer chamber and the impossibility to formulate these two samples. In order to solve this, these points needed to be adjusted to the closest feasible values. The resulting experimental set is shown in *Figure 16* and in more detail in *Table 5*.

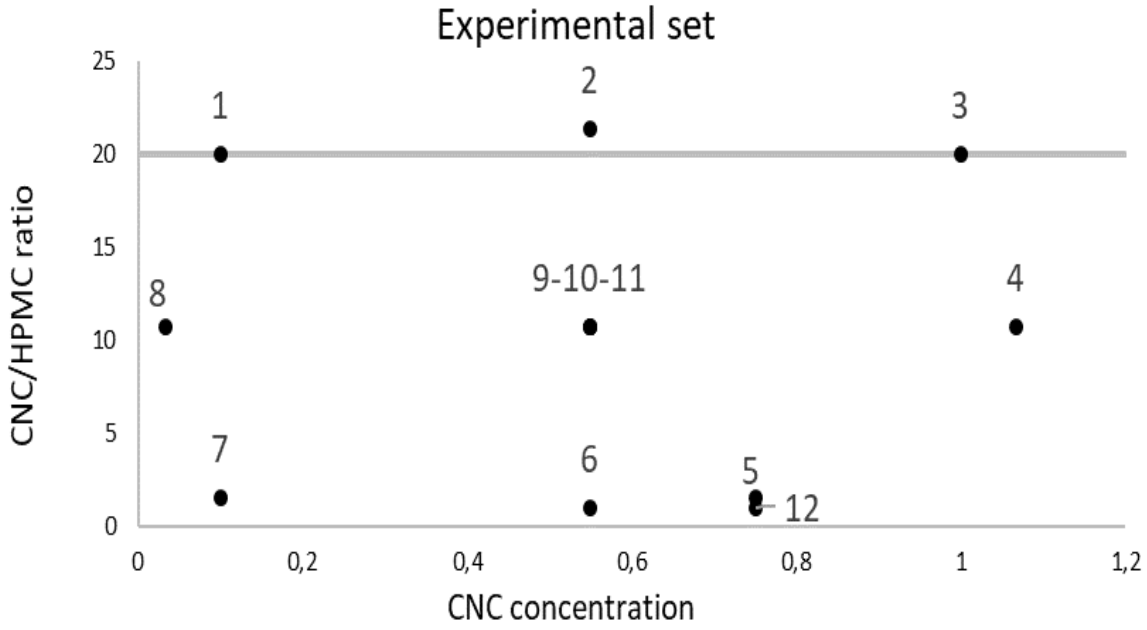

Figure 16. MODDE final experimental set.

Table 7. Experimental set details.

Exp. N°	CNC Conc.	CNC/HPMC	HPMC Conc.
1	0.1	1/0.05	0.005
2	0.55	1/0.047	0.026
3	1	1/0.05	0.05
4	1.1	1/0.09	0.099
5	0.75	1/0.67	0.5
6	0.55	1/1	0.55
7	0.1	1/0.67	0.067
8	0.03	1/0.09	0.003
9	0.55	1/0.09	0.051
10	0.55	1/0.09	0.051
11	0.55	1/0.09	0.051
12	0.75	1/1	0.75

Experiment 12 was subsequently added to further study the behaviour of the system in that range. The HPMC concentration, as determined from the CNC/HPMC ratio, was added into the matrix as an additional factor so that its relative influence in the system could also be analysed.

The model used to fit the data was a multiple linear regression method (MLR). The R^2 values of the different models fitting the different responses are shown in *Table 6*.

Table 8. MODDE R^2 values.

Response	R^2
Droplet size at t=0	0.89
Standard deviation	0.83
Creaming rate	0.33

Viscosity	0.93

5.7. Droplet size characterization

The emulsion drop size was measured by means of laser light scattering using a Mastersizer 2000 (Malvern Instruments). The droplets were supposed to be spherical, with an absorption value of 0.01 and a refractive index equal to the oil one (1.53), unless otherwise stated. The size distribution graphs are presented as volume based and when the whole distribution is not shown the values chosen to synthetically describe it are the D[4,3] or De Brouckere mean diameter and the span. The D [4,3] is a volume-based momentum average which means that the importance of each particle in the average is weighted based on its volume. The span is instead defined as:

$$Span = \frac{D90 - D10}{D50}$$

Where D90, D50 and D10 are percentiles that provide the droplet diameter above which 90%, 50%, and 10% of all droplets can be found.

To drop size of all emulsions was measured right after microfluidization, after 24 h, and 1 week, in order to assess the stability of the emulsion against coalescence. All measurements were conducted in triplicate.
The standard deviation was calculated based on the drop size values ($D_{4/3}$) after microfluidization (d_1), after 24 h (d_2) and 1 week (d_3) by *Equation 2*.

$$Standard\ deviation = \sqrt{\frac{1}{3}\left(\left(d_1 - d_{avg.}\right)^2 + \left(d_2 - d_{avg.}\right)^2 + \left(d_3 - d_{avg.}\right)^2\right)}$$

Equation 2. Drop size standard deviation.

Where:

$$d_{avg.} = \frac{1}{3}(d_1 + d_2 + d_3)$$

Equation 3. Drop size average.

5.8. Surface coverage

The theoretical coverage of the oil droplets by CNCs can be calculated knowing the average dimensions of the crystals and the diameter of the droplets, together with the amount of oil and CNCs used, following *Equation 4.* The result of this equation is expressed in percentage.

$$C = \frac{mD}{6h\rho V}$$

Equation 4. Surface coverage of the oil droplets [40].

Where m is the mass of CNCs in the system (g), D is the D[4,3] of the emulsion droplets (μm), h is the nanocrystals average height (μm), ρ is the density of the CNCs (g/cm^3) and V is the volume of oil in the system (cm^3).

5.9. Interfacial tension

Interfacial tension as a function of time was measured by the pendant drop method with an optical contact angle measuring and contour analysis systems OCA40 (DataPhysics Instruments). Prior to the measurement, a sample of Miglyol was added to an acrylic cuvette of 10 (L) × 10 (W) × 45 (H) mm, and a pendant drop of the aqueous phase solution was formed on the end of a stainless steel needle immersed in the Miglyol oil phase. The aging of the interface can be accurately monitored by measuring the interfacial tension of a drop having a constant interfacial area over a determined number or runs. Typically, the data collected after 250 number of runs were used as the equilibrium interfacial tension values. Three replicate measurements were done for each sample and the data were averaged.

5.10. Viscosity

The flow curves (e.g. viscosity vs. shear rate) of formulations were determined using a rotational shear Kinexus Rheometer (Malvern Instruments) equipped with a 1/40 cone-plate geometry (diameter 40 mm). All measurements were performed at ambient temperature. For the measurements, the samples were transferred onto the lower plate of the rheometer cell and then the upper plate was moved down until a gap of 0.15 mm was obtained. The samples were typically allowed to stand like this for ca. 1 min (stabilizing time) before the measurements were performed. The viscosity at 100 s^{-1} shear rate was extracted from the flow curves of the different formulations (freshly prepared).

5.11. Emulsion creaming

Information on the creaming speed under non-accelerated conditions was determined by means of an optical scanning analyser Turbiscan® Ageing Station (TAGS) (Formulaction) (see details in the *Appendix*). The heart of the Turbiscan® is a detection head which moves up and down along a flat-bottomed cylindrical glass cell. The detection head is composed of a pulsed near infrared light source (880 nm) and two synchronous detectors. The transmission detector (at 180°) receives the light, which goes through the sample, while the backscattering detector (at 45°) receives the light scattered backward by the sample. The detection head scans the entire height of the sample, acquiring transmission (%T) and backscattering (%BS) data every 40 μm (*Figure 17*). Backscattering profiles are used to extract stability information in opaque samples, while transmission profiles are mainly used for the analysis of clear or semi-transparent samples. The TAGS allows for a fully automated analysis of the samples.

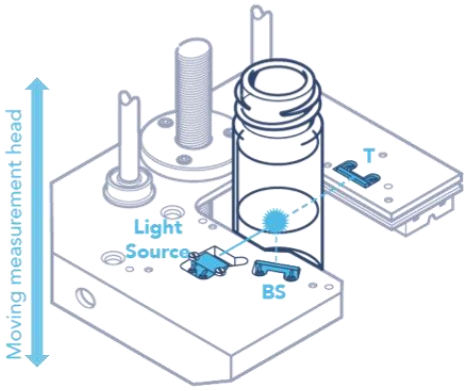

Figure 17. Scheme of Turbiscan instrument [46].

The samples were scanned every 1 h for 24 h, then once a day for one month and finally once a week until the end of the project. The creaming rates are defined as difference in transmission (ΔT) per day. They have been calculated by taking the transmission curves (see *Appendix* for more details) given by the Turbiscan^{LAB} in the range 0.5-7 days (*Figure 18*) and by following *Equation 5.* The result was expressed in units of %T over days.

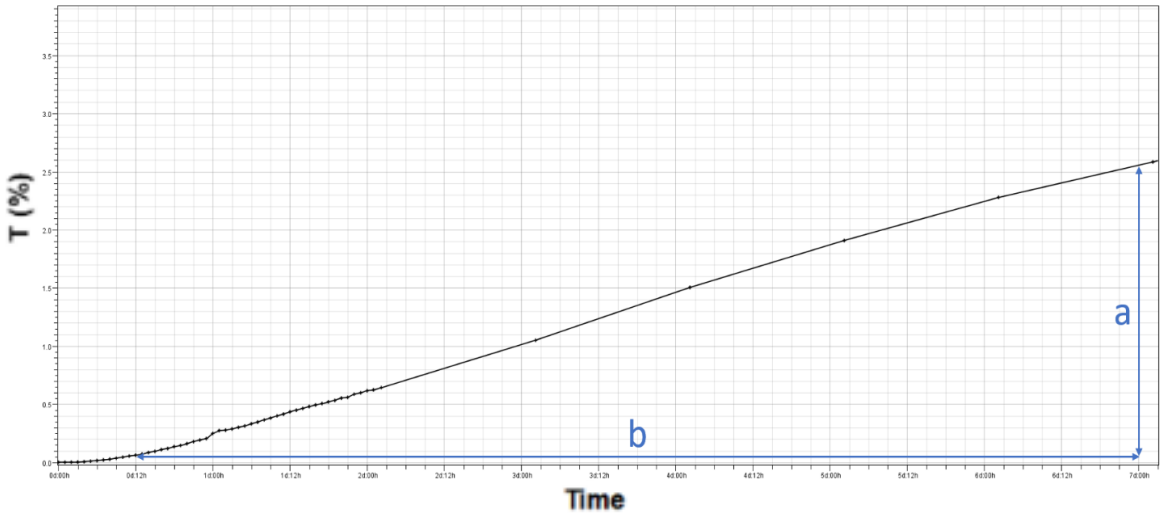

Figure 18. Mean transmission values.

$$Creaming\ rate = \frac{a}{b} \quad [\Delta T/day]$$

Equation 5. Creaming rate formula.

The mean transmission values plotted in *Figure 18* were calculated by the Turbiscan^{LAB} software from the bottom of the sample vial up to 28 mm above.

Consideration on creaming stability have been made also by visual appearance of the formulations. Pictures were taken right after microfluidization, after 24 h and after 1 week for all samples.

5.12. *In vitro* permeation tests

In-vitro permeation tests were performed with the Franz-cells diffusion cells (PermeGear), whose set-up can be seen in *Figure 19*. In the process of identifying the most suitable set-up

(membrane material and pore size, receptor fluid composition and composition of active in the donor formulation) a series of membranes were used (*Table 7*).

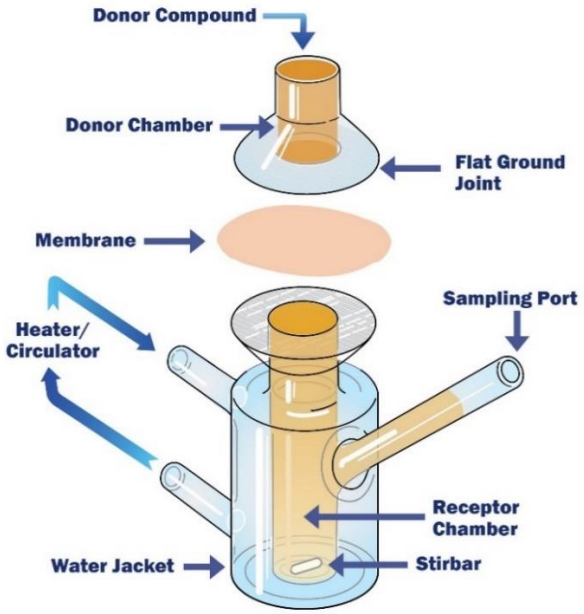

Figure 19. Franz cell set-up [44].

The best performing set-up consisted of:

i) Biomax (polyethersulfone) ultrafiltration discs (Millipore) of 44 mm diameter as membrane.
ii) Aqueous solution with 70% ethanol and 2% Tween 80 as receptor fluid.
iii) Lutein concentration in the formulation of 0.5 wt.%.

A mass of 200 mg of formulation (containing 1 mg of lutein) was introduced in the donor side on a membrane area of 0.64 cm^2. The receptor fluid had a volume of 5 mL and 200 µL of it was extracted by the sampling port every hour for the first 6 hours and then once after 24 hours. Lutein concentration was measured by UV-vis spectrophotometry at 445 nm. The experiments were run on triplicates and at a temperature of 35 °C.

Table 9. Franz cell membranes characteristics.

Membrane	Material	Pore size [µm]
Hydrophilic	Cellulose acetate	0.45
Hydrophilic	Mixed cellulose esters	0.22
Hydrophilic	Cellulose nitrate	0.10
Inorganic	Alumina matrix	0.02
Hydrophilic	Polycarbonate	0.015
Hydrophilic	Polyethersulfone	0.006

To calculate lutein concentration in the receptor fluid a standard calibration curve was used. A mass of 1 mg of lutein was dissolved in 5 mL of the optimized receptor fluid (70% ethanol, 2% Tween 80). This solution of 200 mg/L of lutein was further diluted to values of 8 mg/L, 4 mg/L, 2 mg/L and 1mg/L and the absorbance of these sample at 445 nm was measured. The result is the standard calibration curve shown in *Figure 20.*

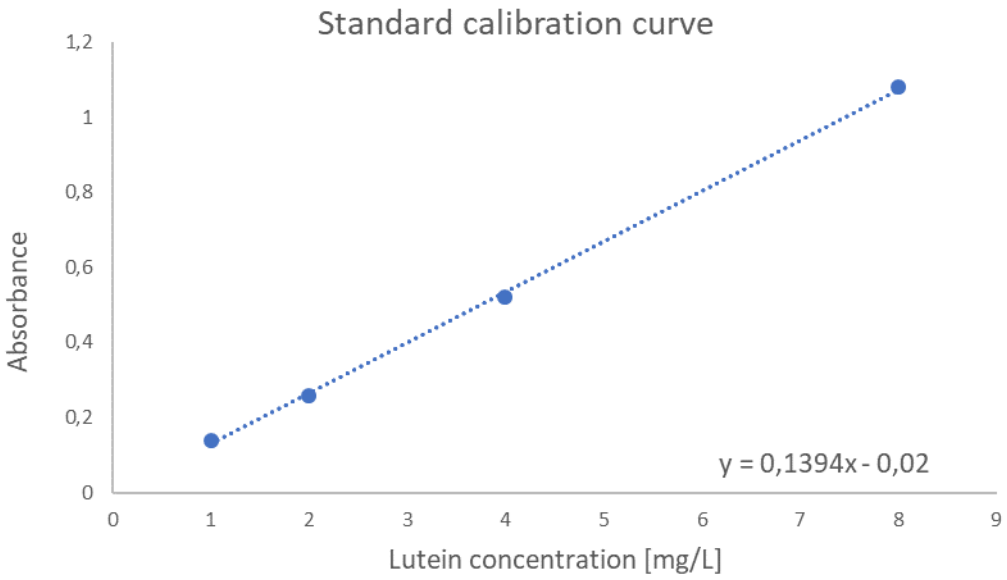

Figure 20. Standard calibration curve for lutein absorbance in the receptor fluid at 445 nm.

The Franz cells results are shown as cumulative amounts. These represent the total amount of lutein permeated through the membrane at t = i and have been calculated following *Equation 6*.

$$Cumulative\ amount\ of\ lutein = c_{t=i} * 5\ mL + c_{t=i-1} * 0.2\ mL$$

Equation 6. Cumulative amount of lutein formula.

Where $c_{t=I}$ is the lutein concentration of the sample extracted at t = i and $c_{t=i-1}$ is the lutein concentration of the sample extracted before the one at t = i.

6. Results and discussion

6.1. Characterization of cellulose nanocrystals

The properties of the CNCs used in this work are summarized in *Table 8.*

Table 10. Key CNC physical and chemical properties.

Property	Value	Technique
Particle Size	z-avg.d = 83 nm	Dynamic Light Scattering
	Length = 75-214 nm Height = 3-10 nm Aspect ratio = 24	Atomic Force Microscopy
Zeta Potential	ζ = -28.1 ± 0.8 mV	Electrophoretic Mobility based on Light Scattering
Surface Charge Density	226 ± 2 mmol/kg	Conductometric Titration

Due to the high aspect ratio of the CNCs, the z-avg. diameter shown in *Table 8* represents an "apparent particle size", since a spherical shape of the particles is assumed by the instrument in the calculations.

The result of the AFM imaging of the CNCs is shown in *Figure 21*, showing the characteristic rod-shape of the nanocrystals.

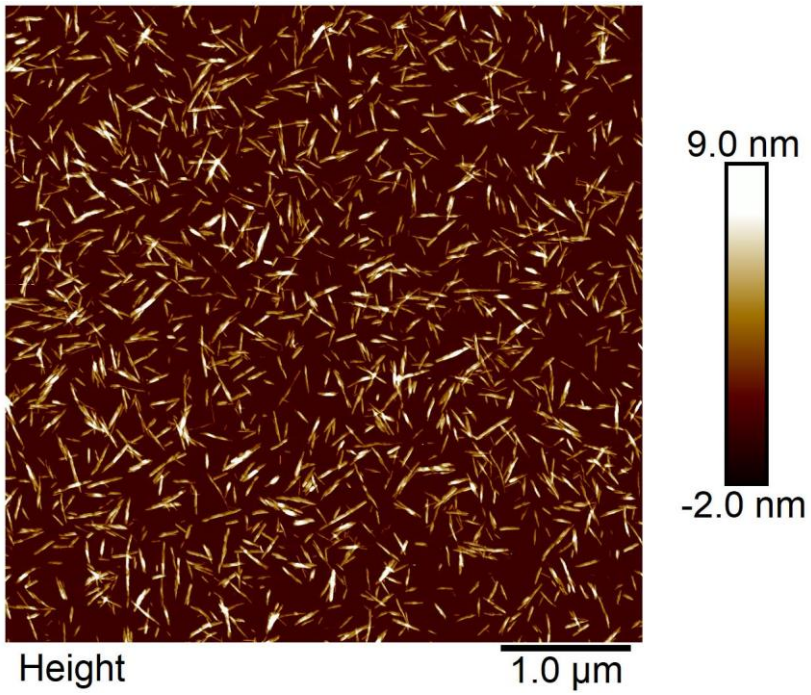

Figure 21. AFM height image of CNCs dried on a silicon wafer from a dilute stock suspension.

6.2. Formulation study

The aim of this section is to evaluate the effect of changing CNC and HPMC concentration on emulsion drop size and stability. Explanations on how each of the two compounds contributes to the emulsion's properties will also be presented. It is important to notice that, as shown in *Figure 16*, all experiments were conducted with a CNC/HPMC ratio lower than 1:0.05 (with

experiment 2 being the only exception). This means that, unless otherwise stated, when talking about CNCs, it will have to be intended as HPMC-modified CNCs, and changing the HPMC amount will mean to change the amount of excess HPMC in the system.

6.2.1. Droplet size at t = 0

6.2.1.1. 50 wt.% oil emulsions

The results of this matrix of experiments can be seen in *Table 9*.

Table 11. Experimental set drop size data.

Exp. N°	CNC conc. [wt.%]	CNC/HPMC	HPMC conc. [wt.%]	$D_{4/3}$ [μm]
1	0.1	1/0.05	0.005	132
2	0.55	1/0.047	0.03	73
3	1	1/0.05	0.05	19
4	1.07	1/0.09	0.01	15
5	0.75	1/0.67	0.5	2
6	0.55	1/1	0.55	1.83
7	0.1	1/0.67	0.07	104
8	0.03	1/0.09	0.003	152
9	0.55	1/0.09	0.05	42
10	0.55	1/0.09	0.05	41
11	0.55	1/0.09	0.05	43
12	0.75	1/1	0.75	1.68

Surprisingly, all diameters measured were still above 1 μm, with a minimum of 1.68 μm reached at higher CNC and HPMC concentrations (0.75%). On the other hand, it is important to note that this value is more than 20% lower than what has been reported by Hu et al. [39] for the CNC + MC system with the same CNC concentration and 45% lower of the CNC + HEC system. The difference can be explained by two different reasons. First, the presence of a slightly different cellulose derivative, which is HPMC, and secondly, the use of a more powerful emulsification technique, i.e. microfluidization, instead of probe sonication.

However, despite these changes, sub-micron diameters are still far from being reached. The reason why this happens is therefore investigated in rest of this section.

First, the results from *Table 9* can be visualized in what is called a MODDE "contour plot". Here the dependence of, in this case, droplet size at t=0 is expressed as a function of the two variables of the system: CNC and HPMC concentration (*Figure 22*). It is important to note

that the values in the contour plot are not the experimental values, but the ones predicted by MODDE software by multiple linear regression of the experimental data. The fitting of the data is instead shown in *Figure 23* and *Figure 24*.

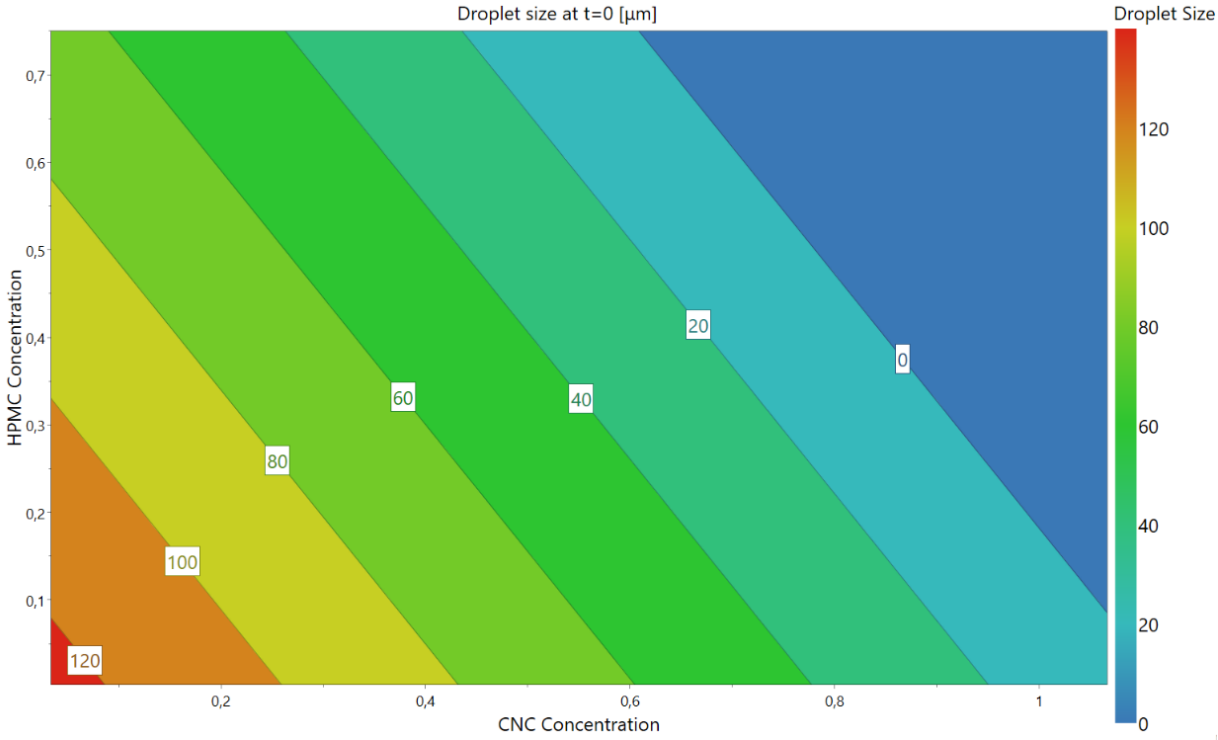

Figure 22. MODDE contour plot of drop size at t=0 (50% oil in water emulsion).

The fact that the areas with the same colour, i.e. same diameter, in the contour plot are neither completely vertical nor completely horizontal suggests that the droplet size of the emulsions is dependent on both CNC and HPMC concentrations, also confirmed by *Figure 23* and *Figure 24*. This further motivates the importance of this study, as only CNC concentration changes had been studied previously in the literature.

Experimentally it has been shown that increasing CNC concentration reduces droplet size and this trend is further confirmed by *Figure 23*. What is more interesting is that increasing HPMC concentration also seems to produce the same affect (*Figure 24*). The reasons behind these two trends need to be explained to see whether further optimization of the system could lead to smaller drop diameters. To do that, the effect of CNC and HPMC were studied separately.

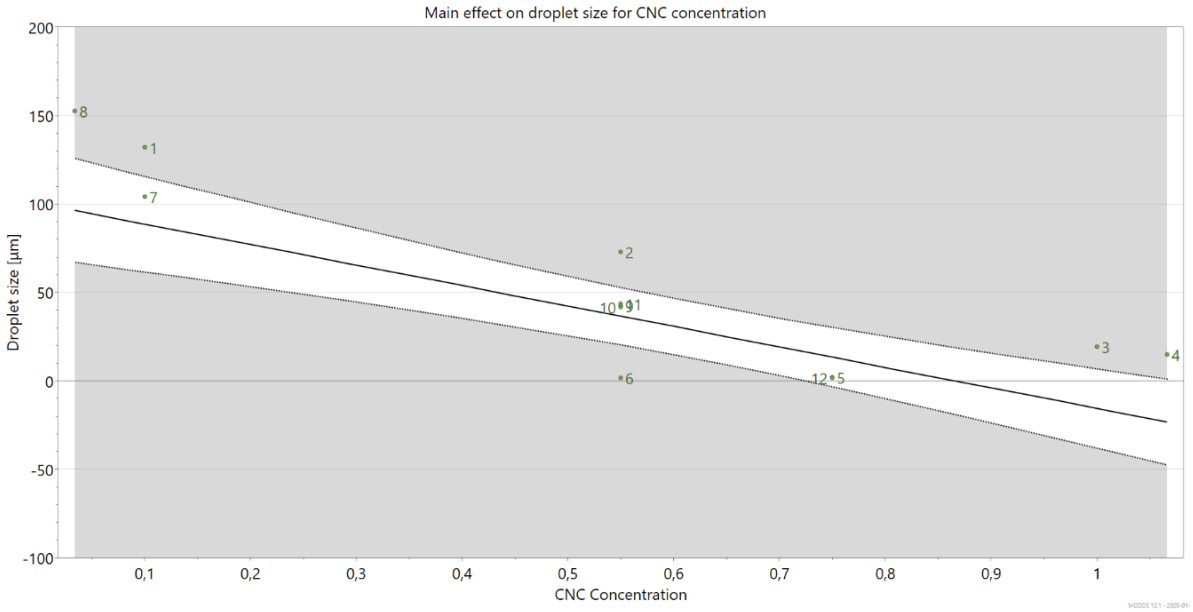

Figure 23. Main effect of CNC concentration on drop size at t=0.

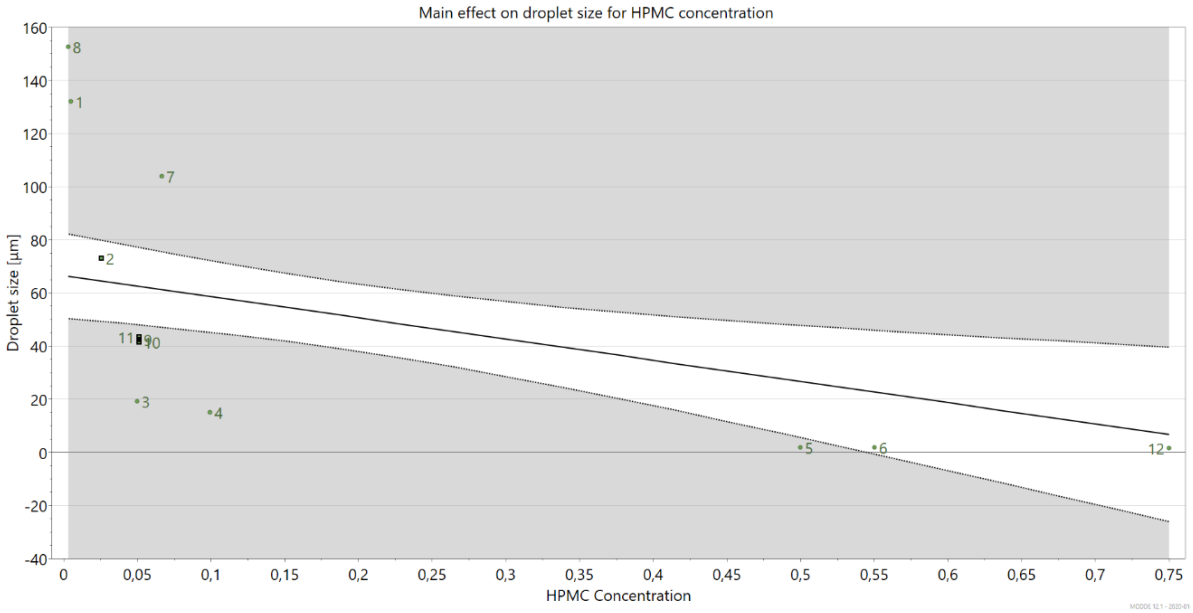

Figure 24. Main effect of HPMC concentration on drop size at t=0.

Effect of CNCs on droplet size

The reason behind the decrease in droplet size when increasing CNC concentration is, at first, quite intuitive. More CNCs means more material to be able to stabilize a larger interfacial area. However, this reasoning is not always valid. It is true when the lack of emulsifier is the only obstacle that prevents the stabilization of smaller droplets. Going back to the scheme reported in *Figure 6* and re-proposed here in *Figure 25,* it could be said that having a sufficient amount of CNCs is essential in preventing the newly formed droplets to coalesce just after microfluidization. On the other hand, if smaller drops were not able to be formed in

the high shear zone of the microfluidizer, increasing CNC will not influence the break-up process and the drop size will not see any change.

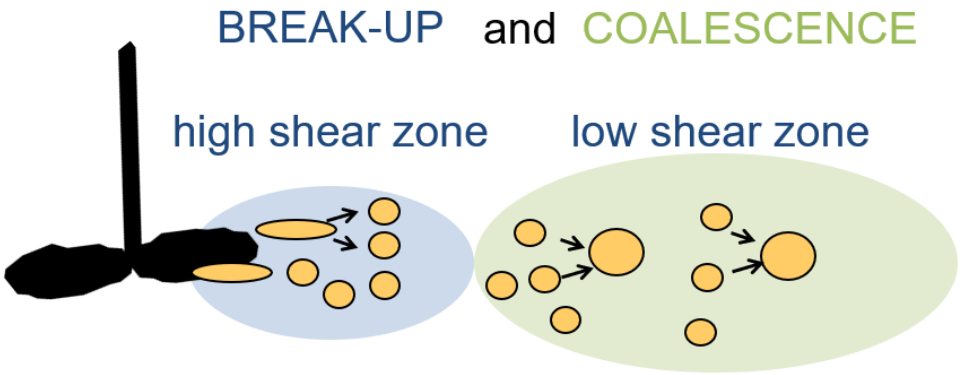

Figure 25. Scheme of droplet break-up and coalescence in the emulsification process.

The surface coverage data, shown in *Table 10,* are useful to further prove this concept. The surface coverage is the percentage of the oil droplet surface that is covered by the CNCs. By looking at the table, the first thing that can be noticed is the presence of 6 values (half of the experimental set) that are above 100 %. What does it mean? This is an indication that in those systems not all CNCs adsorbed at the oil-water interface but remained in excess in the aqueous phase. Smaller diameters could, therefore, have been achieved if smaller droplets would have been formed and quickly stabilized in the high shear zone.

Table 12. Surface coverage data.

Exp. N°	CNC conc. [wt.%]	CNC/HPMC	HPMC conc. [wt.%]	C
1	0.1	1/0.05	0.005	58%
2	0.55	1/0.047	0.03	185%
3	1	1/0.05	0.05	150%
4	1.07	1/0.09	0.01	126%
5	0.75	1/0.67	0.5	22%
6	0.55	1/1	0.55	14%
7	0.1	1/0.67	0.07	63%
8	0.03	1/0.09	0.003	11%
9	0.55	1/0.09	0.05	129%
10	0.55	1/0.09	0.05	134%
11	0.55	1/0.09	0.05	146%
12	0.75	1/1	0.75	18%

At this point it is essential to understand what in the emulsification process can influence the break-up of the droplets. The most common one is the energy input that is given to the system. In this special case, it means the pressure used in the microfluidizer. Unfortunately, the 500 bar pressure used in this project was the maximum pressure achievable in the lab, therefore, it could not be varied. Another factor influencing break-up is suggested by the surface coverage data. If exp. 1-7-8 are excluded as they were unstable due to the very small amount of CNCs, it can be seen that C drops below 100% only if the HPMC concentration in the system is high. The reason why this happens is the focus of the following section.

Effect of HPMC on droplet size

To better understand the effect of HPMC on droplet size it has been useful to visualise the data of *Table 9* only in dependence of the HPMC concentration. Exp. 1-7-8 were excluded from the graph, because they were shown to be unstable due to very low CNC concentration. The graph is shown in *Figure 26*.

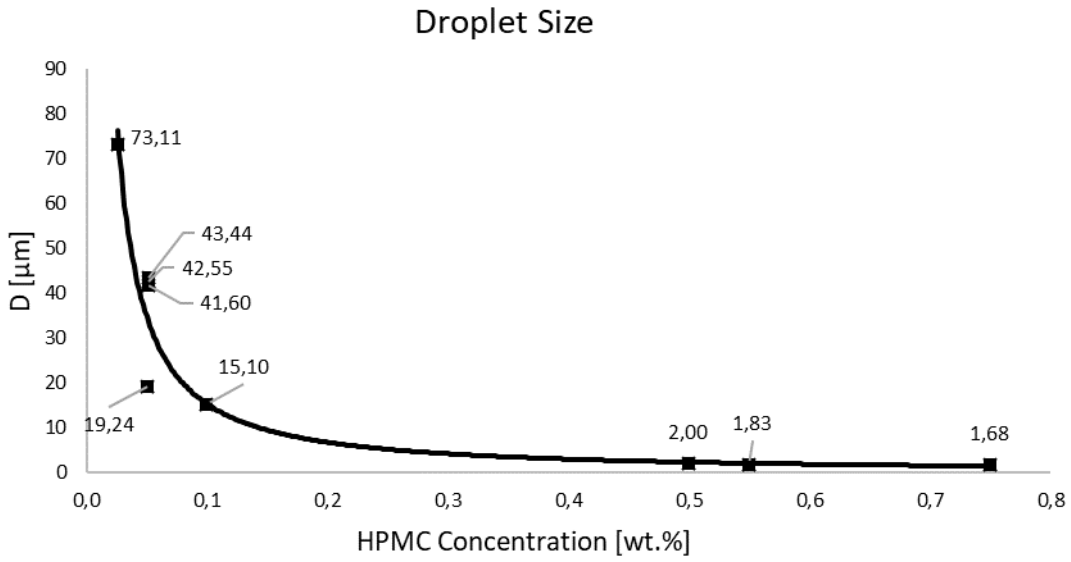

Figure 26. Droplet size vs. HPMC concentration.

The experimental data showed a very good fitting ($R^2 = 0.97$) by power law regression. This allowed to make a clear distinction between two areas of the graph, the one at high HPMC concentration and the one at low HPMC concentration. In the latter, it can be seen how changing the amount of HPMC causes a substantial impact on droplet size (steep slope). The reason, as anticipated, must be related to the ability of HPMC to favour the break-up and fast stabilization of the droplets. This ability is given by two different characteristics of the polymer: surface activity and viscosity.

Surface activity of stabilisers

HPMC has been shown to act as emulsion stabilizer itself without CNCs [43]. Schulz et al. shows the possibility to produce submicron emulsions with only HPMC via microfluidization. However, the droplet diameters were observed to increase after few weeks, which would have required further studies on the stability of these systems. The ability to stabilize o/w emulsions is given by the fact that HPMC itself is a surface-active polymer and therefore is able to both lower the interfacial tension and reach the oil-water interface quickly (and faster than CNCs as a result of the smaller size and faster diffusion). This means that when HPMC is in the system, the breaking-up of the big droplets of the pre-emulsion into smaller ones is easier. Moreover, the stabilization of the newly formed droplets can be faster preventing them to coalesce to bigger ones before they could be reached by the CNCs. The effect of HPMC on interfacial tension has also been quantified by measuring the values for a system with only CNC, one with only HPMC and one with HPMC-modified CNCs with no excess of HPMC. The results are shown in *Figure 27.*

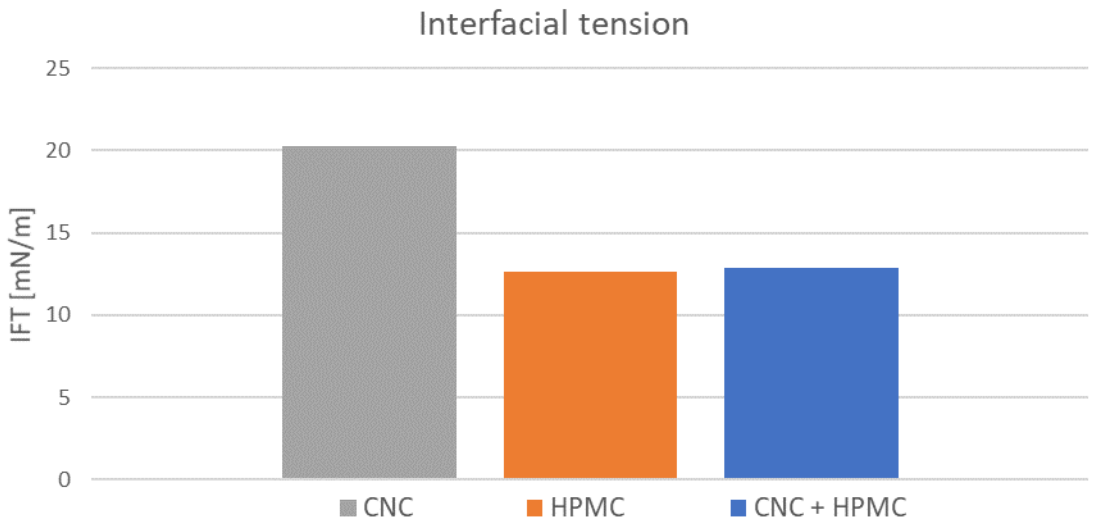

Figure 27. Interfacial tension of different aqueous phase compositions in oil (MCT).

The reduction in interfacial tension is almost of 50% when HPMC is used instead of CNCs and the same value is measured once the CNC are fully covered with HPMC.

A further support the capability of HPMC to allow the system to reach smaller droplet diameters is shown by a comparison that has been performed between two different emulsions with the same amount of stabilizer but in one case unmodified CNCs were used and in the other one only HPMC was used. The difference in droplet size was more than 7 times, with the HPMC emulsion droplets reaching 2.86 µm and the CNC emulsion 20.8 µm.

Effect of stabiliser concentration on viscosity

HPMC has been successfully used commercially as viscosity enhancer [43]. Based on the degree of polymerization, HPMC with different viscosity properties are available. The one used in this project can be considered as medium-high viscosity HPMC; a 2% polymer solution at 20 °C had a viscosity of 4000 mPa s. The values of commercial HPMC are usually included in the range 4 – 15000 mPa s.

By increasing HPMC concentration in the system, the viscosity of the aqueous phase was expected to increase. *Figure 28* clearly shows this effect. The primary conclusion from the graph is that by changing the HPMC concentration, which means moving vertically on the graph, large changes in the viscosity can be obtained. The same thing cannot be stated in the case of CNC concentration. Even large increases in CNC concentration did not increase the viscosity of the aqueous phases.

But how does increasing the viscosity of the aqueous phases relate to the formation of smaller droplets? The answer is that higher viscosity means faster transfer of the momentum in the microfluidizer, and therefore a higher effectiveness in breaking up the oil droplets. However, if the viscosity is too high, however, this is not favourable either, as it could lead to the opposite desired effect.

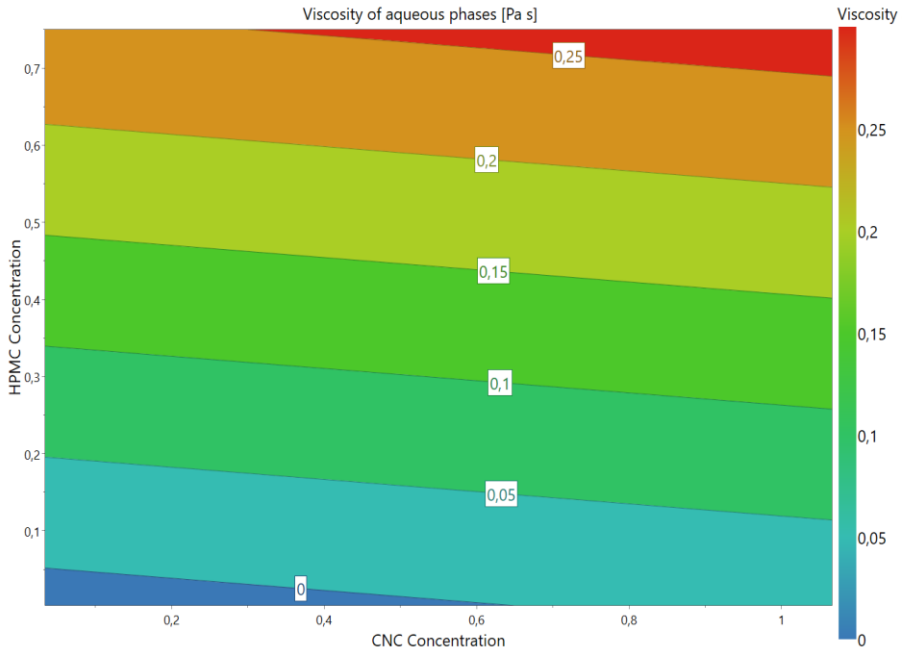

Figure 28. MODDE contour plot of viscosity of aqueous phases at 100 s^{-1}.

So far, we have shown the impact of HPMC on droplet size at low concentration. However, it is still not clear why at high concentration its effect is almost negligible. In fact, coming back to *Figure 26*, the curve slope at high HPMC concentration is almost flat. The interpretation here is that the problem could have shifted to the lack of emulsifier in the system, avoiding immediate coalescence of the newly formed droplets *(Figure 25)*. In this case, smaller droplets could be created but there was insufficient CNCs to stabilized the new oil-water interface created. The calculated values for surface coverage of the data points in this region of the graph could further validate this hypothesis (*Table 11*).

Table 13. Emulsion surface coverage by CNCs (approximate calculation) at high HPMC concentration.

Exp. N°	CNC conc.	CNC/HPMC	HPMC conc.	C
5	0.75	1/0.67	0.5	22%
6	0.55	1/1	0.55	14%
12	0.75	1/1	0.75	18%

All values are very close to the 20% that Hu et al. [39] found to be the minimum coverage value to form stable droplets with a CNC/HPMC ratio of 1/0.67.

At this point, it could be suggested to increase the amount of CNC in the system maintaining the same CNC/HPMC ratio in order to still provide the beneficial effects of HPMC in reducing the droplet diameter. However, this is not physically feasible as the presence of both CNC and HPMC at high concentration causes the clogging of the microfluidizer chamber, due to the high viscosity. Since not enough CNCs were present to stabilize the oil as smaller droplets, a decrease in the amount of oil was investigated, so that we could keep the same concentration of CNCs and HPMC (and same ratio) but would have a much higher CNC/oil ratio.

6.2.1.2. 10 wt.% oil emulsions

The same matrix of experiments was then repeated in the case of a 10% oil emulsion. The results in terms of droplet sizes at t=0 are reported in *Table 12*.

Table 14. Experimental data set of droplet size for 10% oil in water emulsions.

Exp. N°	CNC conc. [wt.%]	CNC/HPMC	HPMC conc. [wt.%]	$D_{4/3}$ [µm]
1	0.1	1/0.05	0.005	13
2	0.55	1/0.047	0.03	6.81
3	1	1/0.05	0.05	6.02
4	1.07	1/0.09	0.01	1.21
5	0.75	1/0.67	0.5	0.617
6	0.55	1/1	0.55	0.588
7	0.1	1/0.67	0.07	2.84
8	0.03	1/0.09	0.003	11.5
9	0.55	1/0.09	0.05	2.12
10	0.55	1/0.09	0.05	2.64
11	0.55	1/0.09	0.05	2.12
12	0.75	1/1	0.75	0.696

The hypothesis introduced in the previous section was then validated. Providing the system with more CNCs with respect to the amount of oil, allowed submicron droplet diameters to be achieved with a minimum size of 588 nm at the same optimum conditions as with the 50% oil emulsions. This represent, as far as we know, the smallest droplet sizes achieved to date with CNCs as stabilizers in o/w emulsions. Compared to Bai et al. [29], there has been a reduction of more than 70% in droplet size for a formulation with the same CNC concentration and a similar value of the microfluidization pressure (600 bar). The difference goes down to 40% if their optimum system is considered with the same CNC concentration but with more than double the pressure (1200 bar).

Furthermore, the same trends discussed in the previous sections were observed again in the case of the 10% oil study, but only shifted to smaller diameters. An example is shown in *Figure 29*. Here, it can be seen that the data points are 12, compared to the 9 of the 50% oil study. This is because the three samples with a very low CNC concentrations were not unstable because the amount of oil in this case was 5 times lower than before.

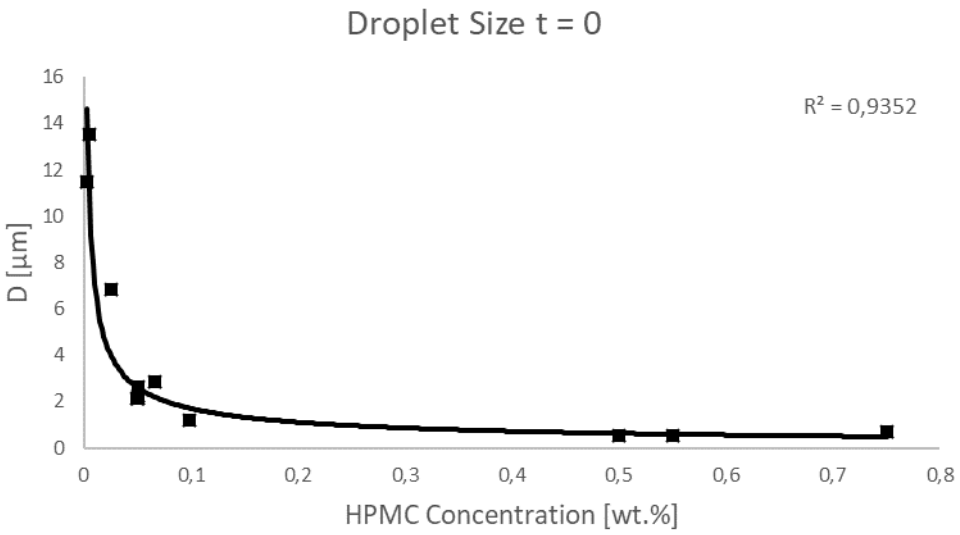

Figure 29. Droplet size vs. HPMC concentration 10% oil.

Despite reaching sub-micron droplet diameters with only 10% oil in the emulsion, for the rest of the project all consideration on stability and drug delivery will be made on the 50% oil experimental set. This is due to the fact that emulsions with higher oil content are of more interest for cosmetic/pharmaceutical companies.

6.2.2. Stability

6.2.2.1. Coalescence

Together with droplet size, the stability of the emulsion against coalescence is important to formulate a product that could be of interest for the market. Many literature examples show that smaller droplet sizes are associated with better stability of emulsions. However, this concept needs to be tested in our system. Smaller dimensions of the droplets allows for a slower creaming rate (Stokes law, *Equation 1*) and therefore it allows the droplets to remain far from each other and prevents coalescence. If the emulsifier is not preventing the formation of the bridge between the drops, then they will eventually coalesce even if small. This instability phenomenon is therefore dependent primarily on the stabilizing properties of the emulsifier.

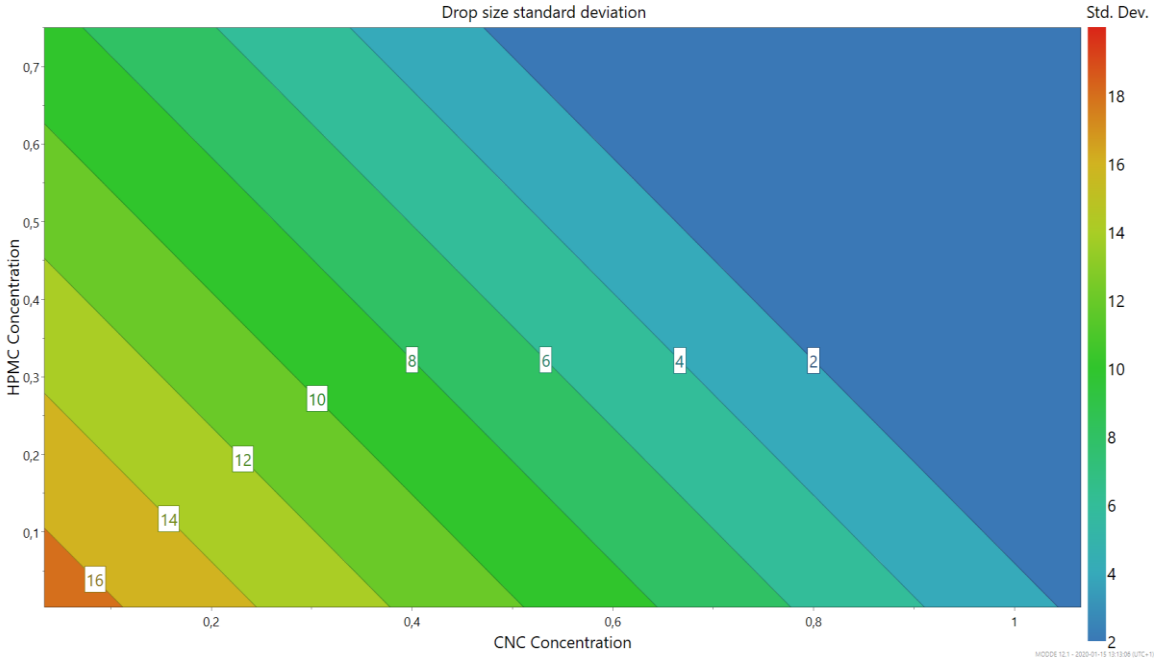

Figure 30. MODDE contour plot standard deviation of droplet size (t=0, 24 hr and 1 week).

CNC stabilised emulsions are commonly known to be stable towards coalescence thanks to their strong absorption to the oil droplets and their steric and electrostatic repulsion. In *Figure 30*, it can be seen that, indeed, increasing CNC concentration decreases the standard deviation, which means an increase in emulsion stability. When CNCs are in high concentration, but HPMC concentration is low, no substantial changes were seen in the mean diameter and its distribution after two months (*Figure 31*).

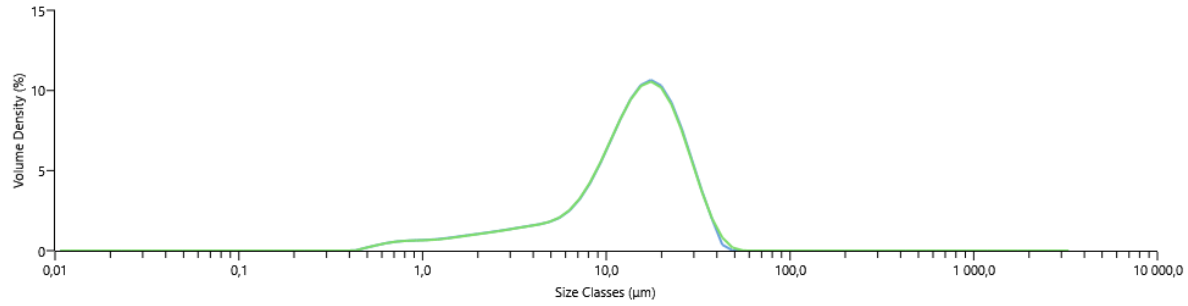

Figure 31. Drop size distribution at t=0 (blue line) and after 2 months (green line) of exp. 4 (1.06% CNC conc., CNC/HPMC=10.75).

On the contrary, when both CNC and HPMC concentrations are low (right-bottom side of *Figure 30*) the standard deviation is high and the systems in that region undergo coalescence with a shift in the whole drop-size distribution (*Figure 32*).

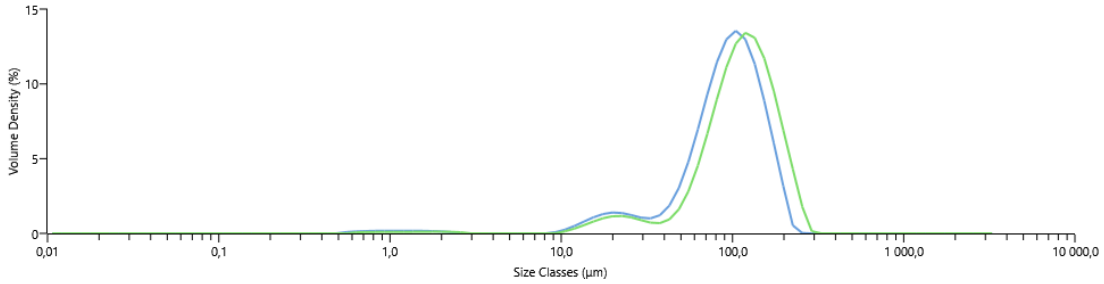

Figure 32. Drop size distribution at t=0 (blue line) and after 2 months (green line) of exp. 7 (0.1% CNC conc., CNC/HPMC=1.5).

Looking at *Figure 30*, it could be observed that a small standard deviation is also achieved in the case when both CNC and HPMC concentrations are high. If the drop size distributions is considered, it can be seen that some perturbations occur at higher drop sizes. In *Figure 33*, the drop size distribution of the optimum case (exp. 12) in terms of dimensions of the droplets is shown. droplets with larger diameters seem to be detected by the instrument and their presence seems to grow over time.

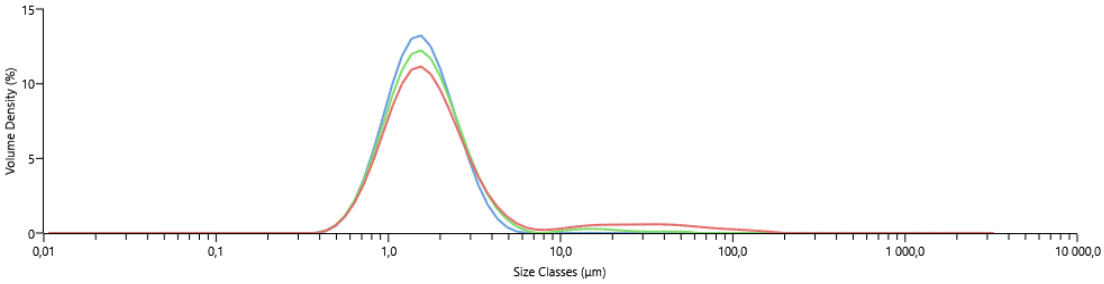

Figure 33. Drop size distribution at t=0 (blue line), after 1 week (green line) and after 2 months (orange line) of exp. 12 (0.75% CNC conc., CNC/HPMC=1).

There are two main explanations for this phenomenon. One is that the large amount of HPMC in the system stabilizes on it's own a small number of droplets. Being HPMC not a good stabilizer this could bring to coalescence over time, which however would not create a shift of the whole distribution. On the other hand, the high presence of HPMC in the system could facilitate the aggregation of small droplets into bigger clusters, that are not broken by the mixer present in the Mastersizer. In order to evaluate which one of the two hypotheses could be more probable, two different observations were made. First, the emulsions were diluted and visualised with an optical microscope (ZEISS Microscopy). Some bigger droplets remained as individual droplets (*Figure 34*), but not consistently on different spots of the sample.

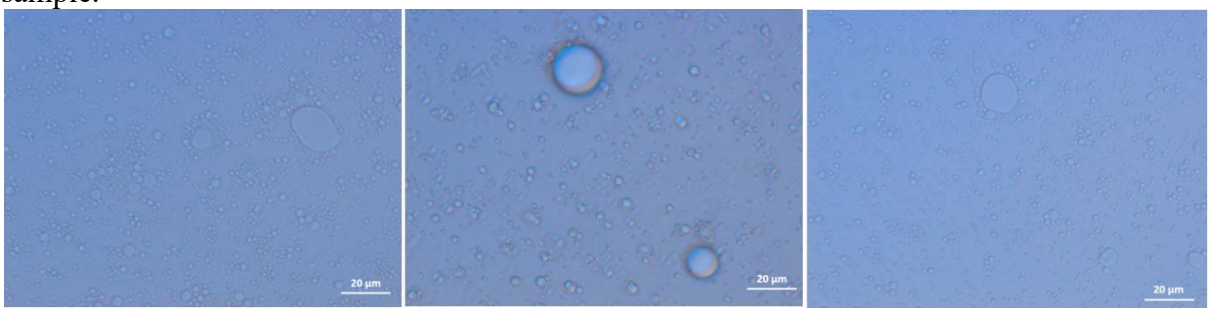

Figure 34. Optical microscope pictures of diluted exp. 12 (0.75% CNC and CNC/HPMC = 1) (scale bar = 20 µm).

Furthermore, the emulsions were pre-diluted and vortexed before being introduced into the Mastersizer. If agglomerates were present in the system this should have broken them apart. Unfortunately, this technique didn´t show any difference as the distribution remained the same.

6.2.2.2. Creaming

To have a qualitative idea of the different formulations a visual evolution of the emulsions in time is shown in *Figure 35*.

Figure 35. Pictures of all the emulsions at t=0 (top) and after 1 week (bottom).

The effect of the variation of CNC and HPMC concentration on the creaming rates of the emulsions is described by the contour plot in *Figure 36.*

Both the increase in CNC and HPMC concentration reduce the creaming rates and therefore improve the stability of the system towards creaming. The explanation links to Stokes law shown in *Equation 6*.

$$v = \frac{g}{18\ \mu_{cont.ph.}} * \left(\rho_{drop} - \rho_{cont.ph.}\right) * D_{drop}^{2}$$

Equation 6. Stokes law.

Among the different samples, both the viscosity of the aqueous phase ($\mu_{cont.ph.}$) and the droplet size (D_{drop}) change. As it can be seen in *Equation 6*, the two of them contributes to a change in the creaming rate. The higher the viscosity the slower the creaming, but the bigger the droplet size the faster the creaming. By recalling the effect of CNC and HPMC concentration on these two factors (*Figure 22, Figure 28*) some considerations can be made. For example, the low creaming rates at high CNC and HPMC concentrations can be explained by the high viscosity and small drop sizes in that region of the plots. On the contrary, the high creaming rates at low CNC and HPMC concentrations are caused by low viscosity and big drop sizes.

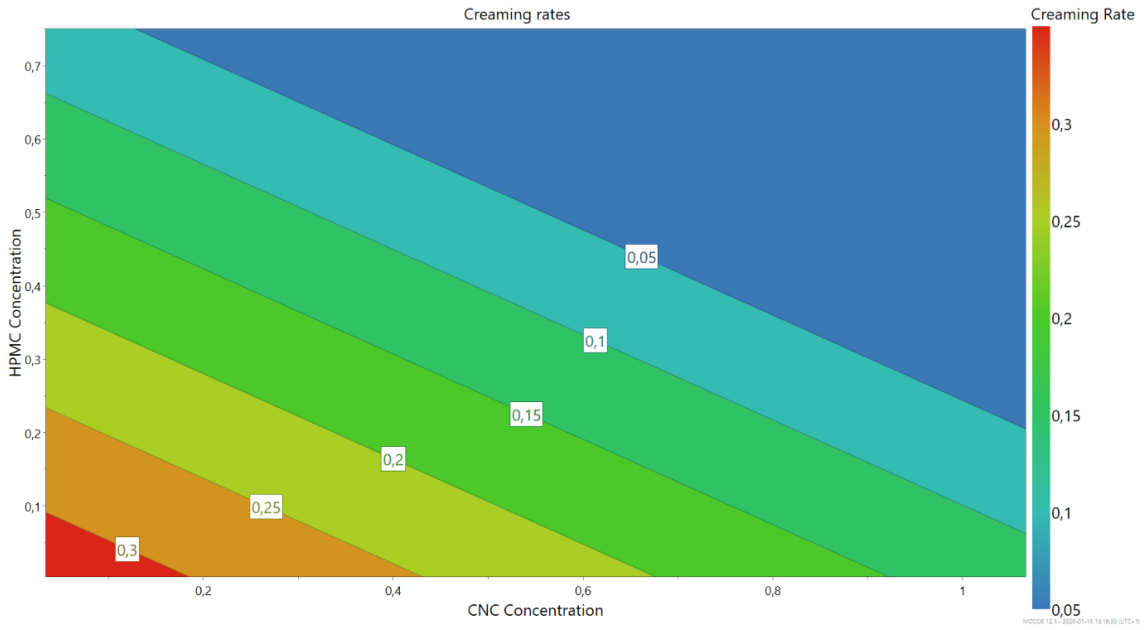

Figure 36. MODDE contour plot creaming rates.

6.2.3. Final formulation for the drug delivery study

High concentrations of CNCs and HPMC have shown to substantially favour the reduction in droplet size and the stability against creaming. However, the high HPMC concentrations seem to provide a negative effect on the drop size distribution of the emulsions, regardless if it is coalescence or formation of agglomerates. The presence of agglomerates would reduce the potential interfacial area of the drug-containing oil droplets with the skin and therefore they are not desirable for cosmetic/pharmaceutical formulations.

Finally, one further experiment was carried out with the goal of trying to achieve a similar drop size as the optimum case for 50% oil emulsion (1.68 µm), but also a better drop size distribution that could be stable over time. To do that, the HPMC concentration was reduced from 0.75% to 0.3%, which gave us the opportunity to double the CNC concentration to 1.5% (no clogging of the microfluidizer occurred). The first experiment was microfluidized at 500 bar resulting in a drop size of 3 µm and a distribution shown in *Figure 37*. The same system could be microfluidized at 900 bar providing a diameter of 2.67 µm and a distribution shown in *Figure 38*. Increasing the pressure together with increasing CNC concentration created a double effect. Higher pressure meant bigger energy input to the system in order to compensate the lack of HPMC in favouring the break-up of the droplets. Furthermore, higher CNC concentration meant an increase in the availability of the stabilizer and its proximity to the newly formed droplets. The most interesting effect was the drop size distribution, which is the main advantage of this system compared to the optimum case. *Figure 39* shows the evolution of the droplet size distribution over two months. The emulsions didn't change at all from t=0 and the monodispersity of the system (span = 0.8) is also of great interest for being a Pickering emulsion.

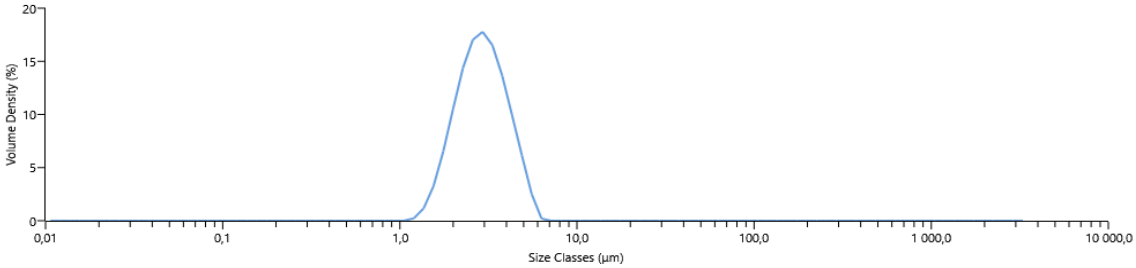

Figure 37. Drop size distribution at t=0 of exp. 13 (1.5% CNC conc., CNC/HPMC=1/0.2).

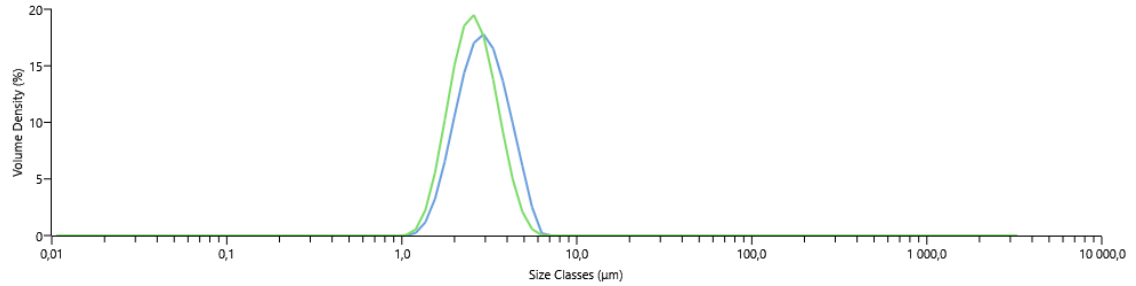

Figure 38. Drop size distribution of exp. 13 (1.5% CNC conc., CNC/HPMC=1/0.2) at 500 bar (blue line) and of exp.13 at 900 bar (green line).

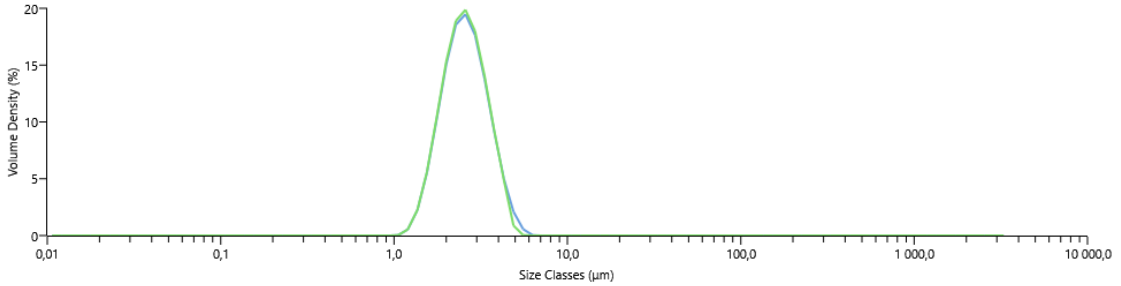

Figure 39. Drop size distribution at t=0 of exp. 13 (1.5% CNC conc., CNC/HPMC=1/0.2) (blue line) and after two months (green line).

The emulsion was also visualized with an optical microscope (ZEISS Microscopy), which further confirmed the monodispersity of this system (*Figure 40*) compared to the one shown in *Figure 34*.

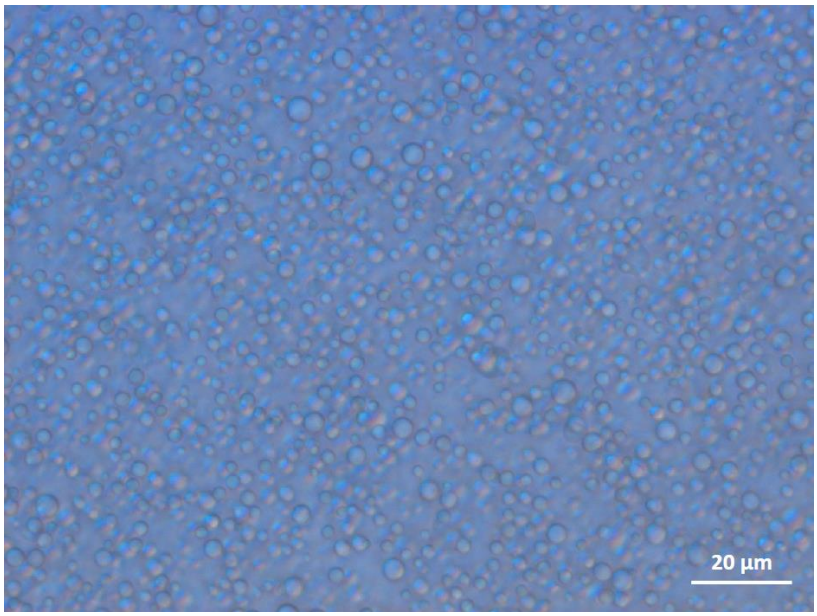

Figure 40. Image of emulsion droplets from exp. 13 (1.5% CNC, CNC/HPMC = 1/0.2) by optical microscope.

6.3. Drug delivery study

6.3.1. Definition of target lutein concentration in oil

Lutein solubility in the MCT oil was investigated. The FloraGlo (20% lutein) was diluted with Miglyol to concentrations ranging from 5% to 0.025%. The resulting solution was visually analysed with the help of filter papers Whatman 5 (GE Healthcare) on which the samples were poured in order to see the presence of undissolved lutein crystals. *Figure 41* shows the qualitative result of the study.

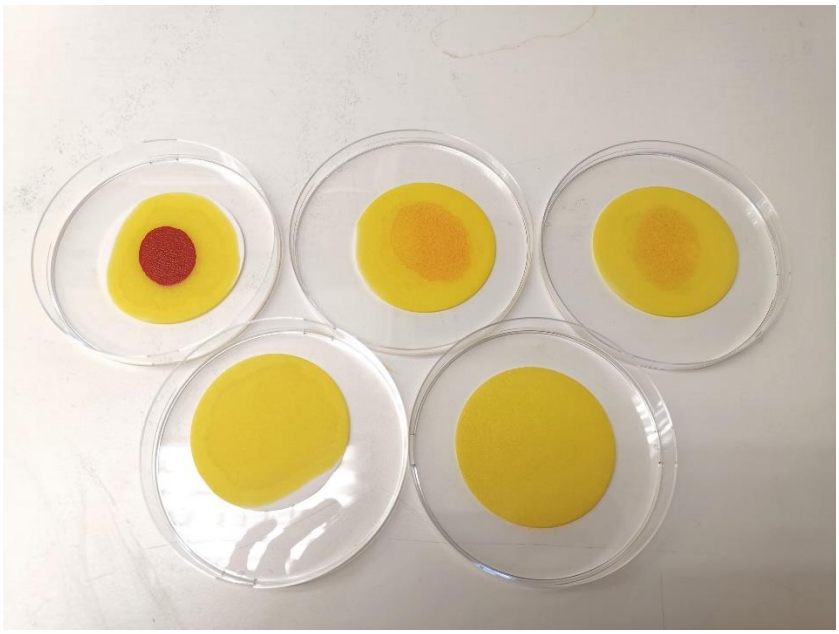

Figure 41. Filter papers with: 5%, 0.2%, 0.1% lutein (upper row, starting from left) and 0.05%, 0.025% lutein (lower row, starting from left).

No crystals were observed on the filter papers below a lutein concentration in the oil of 0.05%.

6.3.2. Franz cell set-up

Paolino et al. [45] studied the permeation of lutein through pig skin using the Franz cells set-up. In this project synthetic membranes composed of cellulose derivatives (cellulose nitrate, cellulose acetate) or synthetic polymers (polycarbonate, polyethersulfone) have been used in alternative to the pig skin. The concentration of lutein in the oil phase was chosen to be equal to the solubility value (0.05%). Unfortunately, none of the membranes showed permeation of lutein when the same receptor fluid of Paolino et al. [45] was used (50% ethanol, 50% water). Therefore, an optimization process of the set-up was undertaken. This involved the testing of different combinations of membranes, receptor fluid compositions and lutein concentrations in the donor side. A common problem during the experiments was the migration of the receptor fluid to the donor side interfering with the formulation and not allowing it to stand alone in the donor side for the whole duration of the experiment (24 hours). An account of the outcome of all these experiments can be found in the *Appendix*, while the main implications of this optimization work are summarised below.

Receptor fluid:

A high amount of ethanol in the receptor fluid was fundamental in creating the solubility conditions necessary as driving force to allow the lutein to move from inside the droplets to the receptor fluid through the membrane.

Pore size of the membrane:

The pore size of the membranes was a key factor in delaying the osmotic transfer of ethanol from the receptor fluid to the donor chamber interfering with the formulation stability. Small pores allowed the system to sit for 24 hours or more.

Membrane material:

Membranes composed of cellulose derivatives such as cellulose nitrate and cellulose acetate showed degradation after a period of only 8-12 hours. The interaction between a highly concentrated solution of ethanol and those membranes did not seem to be compatible. On the contrary, membranes composed of synthetic polymers showed good resistance for at least 24 hours or even more.

Lutein concentration:

Using too low a concentration of lutein in the oil did not allow the permeation of the active component through the membrane unless a high ethanol concentration and a membrane with bigger pore size were used. A higher concentration allowed the use of the membrane with the minimum pore size (6 nm) and an intermediate ethanol concentration of 70%.

A description of the final set-up used for the delivery study in given in *Table 13*.

Table 15. Franz cells final set-up.

Membrane	Hydrophilic Polyethersulfone Pore size: 6 nm
Receptor fluid	70% EtOH 2% Tween80

The characteristics of the different formulations are summarised in *Table 14*.

Table 16. Formulations main characteristics.

Formulation	Drop size [μm]	Viscosity at 100 s^{-1} [mPa s]	Lutein concentration in total emulsion
Lipoid emulsion	2.82	4.98	0.5%
CNC-HPMC emulsion	2.71	520	0.5%
CNC-HPMC pre-emulsion	40	770	0.5%

Drop size and viscosity are two key factors in regulating the permeability of an active through a membrane [30]. Smaller drop sizes mean higher interfacial area available for the active to go from the oil droplets through the membrane in the receptor fluid. Lower viscosity increases the availability of the active close to the membrane, by favouring the diffusion of active-rich oil droplets towards the membrane. In comparing an emulsion stabilized by a classical surfactant used in the food industry, such as the Lipoid, and the optimized CNC-HPMC emulsion, drop sizes and viscosity should, therefore, have similar values. The drop size of the Lipoid emulsion was easily tuned by optimizing the pressure in the microfluidizer and the number of passes. Thus, the values between the two formulations differ of only 11 nm. However, a difference of three order of magnitude is present in the viscosity. Viscosity enhancers in the Lipoid formulation, such as carboxymethyl cellulose (CMC) and HPMC, were used. Unfortunately, the high concentrations needed to cover the difference with the CNC-HPMC emulsion seemed to interfere with the stability of the emulsion.

The purpose of comparing a microfluidized CNC-HPMC emulsions with its pre-emulsion is to evaluate the effect that drop size has on the delivery of an active and eventually support the aim of reaching drop sizes as small as possible. Therefore, a difference in drop sizes is required. However, to be able to make consideration on this comparison viscosities should be similar. In this case the difference is not substantial, but still doesn't allow us to take conclusive considerations on the effect of drop size on effecting drug delivery of the active.

6.3.3. Comparison between CNC-HPMC Pickering emulsions with different droplet sizes

The results of the Franz cells experiments for the CNC-HPMC emulsion and pre-emulsion are presented in *Figure 42*. As it can be seen, the amount of lutein permeated through the

membrane is considerably higher, more than double after 24 hours, for the CNC-HPMC emulsion compared to the pre-emulsion. This could be a validation of what has been shown in the literature, highlighting the importance of reaching small diameters in emulsions used in topical delivery.

Increasing the surface area of the oil droplets can create a sensible change in how fast the active is able to be released from the oil droplets into the continuous phase and permeate through the membrane. However, the difference in viscosities, even if not substantial, doesn't allow to make certain conclusions.

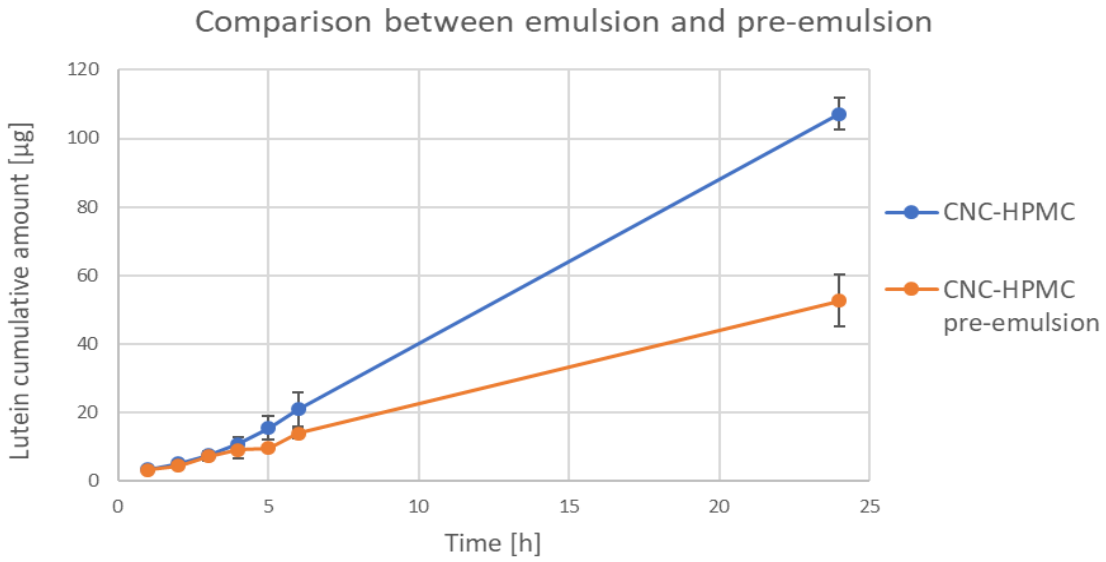

Figure 42. Franz cell results of the comparison between the CNC-HPMC emulsion and the pre-emulsion (1.5% CNC, CNC/HPMC=1/0.2).

6.3.4. Comparison with a classical emulsion

The optimized CNC-HPMC emulsion has also been compared to an emulsion stabilized by a common surfactant (Lipoid S100). The results are shown in *Figure 43*.

As it can be seen, since the first hours the amount of lutein permeating through the membrane is higher for the classical emulsion compared to the CNC-HPMC emulsion. The presence of a densely packed monolayer of nanoparticles at the oil/water interface could act as a barrier regulating the release of the active in the aqueous phase compared to the use of a classical surfactant. This could be beneficial in topical formulations that requires controlled or sustained drug release. However, considerations are difficult to be made on the system, as here the difference in viscosity between the two emulsions is considerable. The rate-limiting step could be the diffusion of the oil droplets from the bulk of the emulsion to the membrane instead of the release of the active from the oil droplets into the continuous phase.

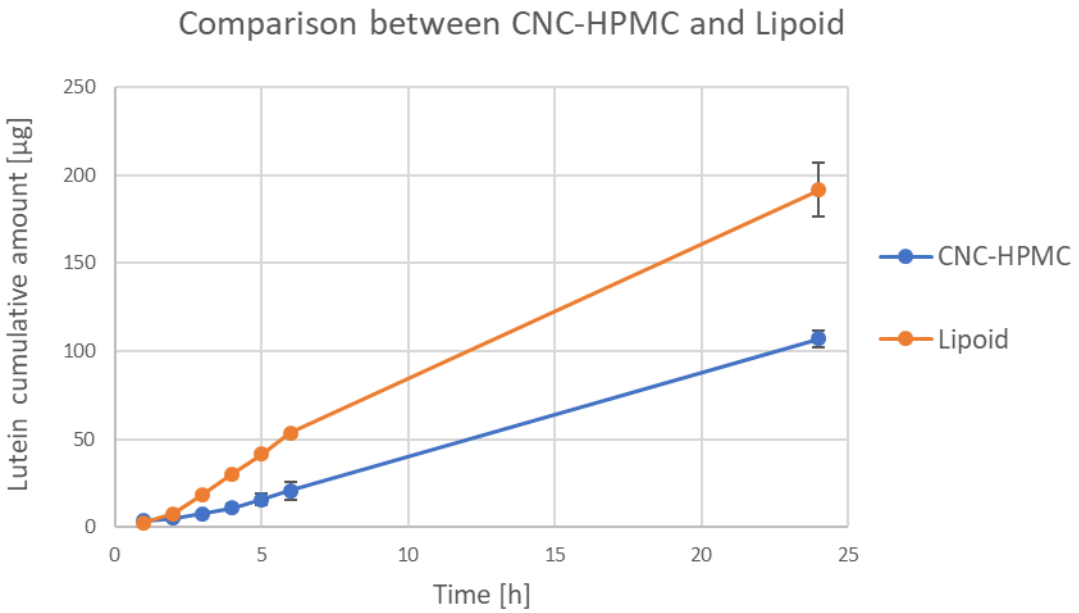

Figure 43. Franz cell results of the comparison between the CNC-HPMC emulsion (1.5% CNC, CNC/HPMC=1/0.2) and the Lipoid emulsion.

7. Conclusions

7.1. Formulation study

- It was possible to produce o/w emulsions with submicron-size drops (average drop diameters down to ca. 600 nm) by means of microfluidization using a combination of CNC and hydroxypropyl methylcellulose (HPMC). The smallest emulsion drop sizes were attained using a microfluidization pressure of 500 bar with a system containing 10% oil, 0.55% CNC and 0.55% HPMC (this is a CNC/HPMC ratio of 1/1 and a CNC to oil ratio of 5/100). Under these concentration conditions, the initial aqueous mixture of emulsifier consisted of both HPMC-modified CNCs (fully covered) and excess HPMC (complete surface coverage of CNCs by HPMC is expected at a CNC/HMPC ratio of 1/0.05).

- Within the CNC and HPMC concentration range studied (0.03% to 1.5% CNC and 0.005% to 0.75% HPMC), high concentrations of CNC in combination with high concentrations of HPMC favoured a reduction of the drop size. For a given microfluidization pressure and a fixed CNC to oil ratio the smallest emulsion drop sizes were attained at a CNC concentration of 0.55-0.75% and a CNC/ HMPC ratio of 1/0.67-1 (i.e. an excess of HPMC). Excess HPMC in the system favours the formation of small emulsion droplets due to its ability to lower the interfacial tension at the oil-water interface and increase the viscosity of the continuous phase of the emulsion. At the same time, in the CNC/HPMC system a minimum amount of CNCs (0.55%) was found to be essential to allow for the stabilization of the newly formed drops and prevent immediate drop coalescence.

- The ability to attain submicron drop sizes at fixed microfluidization pressures is modulated by changes in the CNC to oil ratio on the system. Given the high viscosities of CNC dispersions and HMPC solutions beyond concentrations of 0.75% respectively, changes in the CNC to oil ratio were accomplished by modifying the relative amounts of oil and water in the emulsion rather than by increasing the concentration of CNC and HPMC in the aqueous phase. While the minimum attainable drop size at CNC to oil ratio of 0.75/100 (emulsion containing 50% oil) was 1.68 μm, emulsions with average sizes of ca. 600 nm were achieved at the same CNC concentration and CNC/HPMC ratio when the CNC to oil ratio was 7/100 (emulsion containing 10% oil).

- The presence of an excess of HPMC in the aqueous phase (HPMC concentrations ≥ 0.3%) favoured the stability of the emulsions against creaming, due to the combined effect of having smaller drop sizes and higher viscosities.

- High concentrations of CNC (0.75%) and low HPMC concentrations (< 0.55%), showed good stability towards coalescence for a period of 2 months with no change in drop size distribution over time. High HPMC concentrations (0.55-0.75%) showed, however, either the coalescence of a small part of the droplets, probably those stabilized by only HPMC, or the agglomeration of small drops into larger clusters. By decreasing the HPMC concentration to 0.3% and increasing CNC concentration to 1.5% no coalescence or agglomeration was seen for over 1 month. Furthermore, increasing the pressure from 500 to 900 bar for this system resulted in a very narrow

size distribution (span=0.8) and a drop size of 2.67 μm (1 μm more than the minimum achieved with a 50% emulsion).

7.2. Drug delivery study

- The percentage of lutein permeated trough the membrane in the Franz cells experiments was after 24 hours 11% μg for a CNC-HPMC emulsion and 5% for the non-microfluidized emulsion with same CNC and HPMC concentrations. The difference in drop size from 2.71 μm to 40 μm respectively allowed a faster release of the active, due to a higher surface area available between the droplets and the membrane.

- The classical surfactant emulsion showed a percentage of permeated lutein into the receptor fluid after 24 hours of 19%, 1.7 times more than in the case of CNC-HPMC solution with the same amount of lutein and similar drop size. This could confirm the role of the densely packed CNC-HPMC layer at the oil water interface acting as a barrier, regulating the release of lutein from the oil droplets in what seems to be a more sustained release. The difference of three order of magnitude in the viscosity of the two emulsions does, however, not allow final conclusions.

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Finally, a special thank you to all my friends who have cheered with me in these past 5 months and have contributed to make my Erasmus in Sweden a wonderful experience.

9. Future work

- Further reducing drop-size
  - The full potential of the system could be tested by increasing the CNC concentration (>1.5%) in a 50% oil emulsion and keeping a CNC/HPMC ratio equal or similar to the optimized one (CNC/HPMC=1/0.2).
  - The importance of microfluidizer pressure could also be further studied, as it was shown to help the formation of smaller droplets and produce a much more monodispersed system.
  - Furthermore, a study on the influence of the interaction chambers design on droplet size could also be interesting.
- Robustness and longer-term stability
  - The stability of the best emulsions could be assessed with physical and chemical stresses., spray-drying, freeze-thaw cycles, etc…
- Drug delivery study:
  - A longer time for the drug delivery experiments could also be suggested in order to evaluate whether the amount of active going through is eventually the same.
  - In order to make a fair comparison between the different emulsions in the drug delivery study, the viscosity of the different formulations could be adjusted by optimizing the concentration of a viscosity enhancer that is not interfering with the system. The choice of a synthetic surfactant for the classical emulsion instead of a lecithin (Lipoid S1200) and a non-surface a polymer such as Xanthan Gum could also help limiting the possible interactions between the emulsifier and the viscosity enhancer.
  - The use of real skin such as pig skin could be used in order to understand the effect of the interactions between the CNC-stabilized oil droplets and the skin structure.

10. Appendix

10.1. Refractive index considerations

When measuring the drop size of the 10% oil emulsion the dimensions of the droplets reach values around 500-600 nm that are in the order of magnitude of those of the CNCs (150-200 nm). It was, therefore, investigated if it was appropriate to use the refractive index of the oil instead of that of the particles when measuring drop size with the Mastersizer for those systems. In order to do that the measured data were compared with the predicted ones calculated by the Mie theory used by the Mastersizer. A good fitting means that the refractive index and absorption value given to the software to transform the raw data into a size distribution are correct. An example of a good fitting is shown in Figure 44.

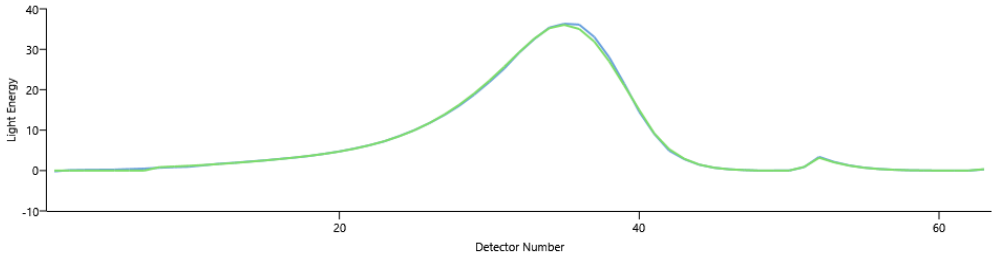

Figure 44. Mastersizer raw data (blu line) and fit data (green line).

The residual is a quantitative evaluation of the fitting of the data. Residual values lower than 1 show a good fitting of the raw data.

In all systems of this project the residuals were found to be lower than 1, except with two 10% oil emulsions providing the lowest diameter of all: exp. 5 (0.75% CNC, CNC/HPMC=1.5) and exp. 6 (0.55% CNC, CNC/HPMC=1). In this case, changing the refractive index from the one of the oil to the one of the particles resulted in a change in the residuals from 1.37 to 0.74 and from 1.32 to 0.79 respectively. The effect on droplet size, however, was not substantial, changing from 533 to 617 and from 532 nm to 588 nm.

10.2. Turbiscan analysis

The transmission values measured by the Turbiscan at every scan along all the length of the sample vial are presented in *Figure 45*.

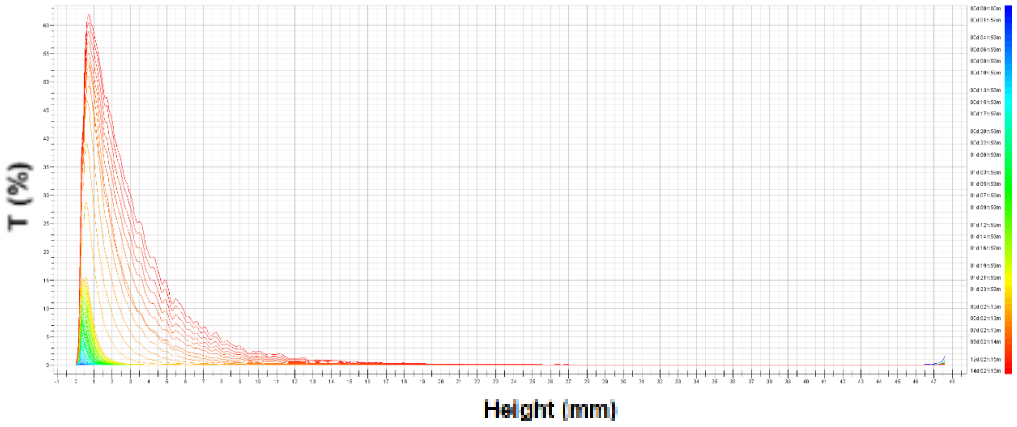

Figure 45. Example of transmission data along the length of a sample over time.

The lines with different colours represent the evolution of the transmission profile of the sample during time. Higher transmission means that the sample in that area is becoming more transparent which in this case means that is undergoing creaming. Low transmission means that the sample is not creaming. The mean values of the transmission can be calculated by an average on an interval of the whole length of the vial. Finally, the result for all samples are the profiles shown in *Figure 46*.

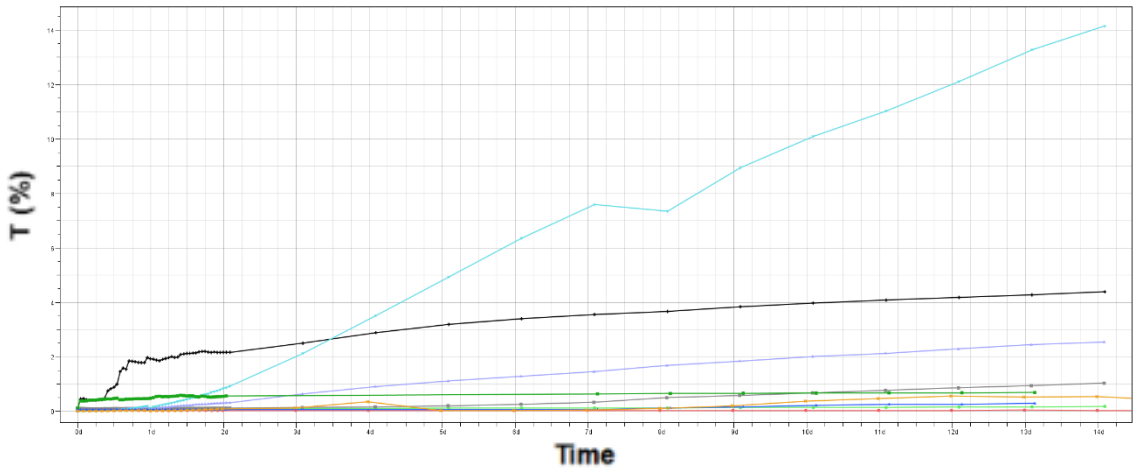

Figure 46. Mean transmission values over time for different samples.

A period of time relevant for all samples can be individuated and the creaming rates can be calculated as shown in *Methods*.

10.3. Franz cells set-up optimization

Membrane	Material	Pore size	Receptor fluid	Lutein conc. in oil	Outcome
Hydrophilic	Polycarbonate	0.015 um	50% EtOH	0.05%	No Lutein through
Hydrophilic	Cellulose acetate	0,45 um	50% EtOH	0.05%	No Lutein through
Hydrophilic	Mixed cellulose esters	0.22 um	50% EtOH	0.05%	No Lutein through
Inorganic	Alumina matrix	0.02 um	50% EtOH	0.05%	No Lutein through
Hydrophilic	Polycarbonate	0.015 um	100% EtOH	0.05%	No Lutein through
Hydrophilic	Cellulose acetate	0,45 um	100% EtOH	0.05%	Lutein through, but EtOH going to the donor
Hydrophilic	Mixed cellulose	0.22 um	100% EtOH	0.05%	Lutein through

	esters				
Inorganic	Alumina matrix	0.02 um	100% EtOH	0.05%	Lutein through, but EtOH going to the donor
Hydrophilic	Mixed cellulose esters	0.22 um	60% EtOH	0.05%	No Lutein through
Hydrophilic	Mixed cellulose esters	0.22 um	70% EtOH	0.05%	No Lutein through
Hydrophilic	Mixed cellulose esters	0.22 um	80% EtOH	0.05%	No Lutein through
Hydrophilic	Mixed cellulose esters	0.22 um	90% EtOH	0.05%	Lutein through, but membrane degradation after 8 hr
Hydrophilic	Cellulose Nitrate	0.10 um	90% EtOH	0.05%	Same as above, but slower transfer of EtOH to the donor
Hydrophilic	Cellulose Nitrate	0.10 um	90% EtOH	1%	Lutein through, but no difference between emulsion and pre-emulsion
Hydrophilic	Polycarbonate	0.015 um	90% EtOH	1%	Lutein though, but no difference between emulsion and pre-emulsion
Hydrophilic	Polyethersulfone	50 kDa	90% EtOH	1%	Lutein through, but no difference between emulsion and pre-emulsion
Hydrophilic	Polyethersulfone	50 kDa	50% EtOH	1%	No Lutein through
Hydrophilic	Polyethersulfone	50 kDa	60% EtOH	1%	No Lutein through

Hydrophilic	Polyethersulfone	50 kDa	70% EtOH	1%	Lutein through and difference between the emulsion and pre-emulsion
Hydrophilic	Polyethersulfone	50 kDa	70% EtOH 2% Tween 80	1%	Lutein through and difference between the emulsion and pre-emulsion

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