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Role of cyclodextrins in biopharmaceutical formulations

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Introduzione

L'utilizzo di proteine in campo farmaceutico è un settore in crescita dal punto di vista della ricerca nel campo dei biofarmaci. Le proteine devono essere analizzate, caratterizzate, formulate e spedite in modo differente rispetto ai farmaci classici, poiché modifiche nella struttura primaria, secondaria, terziaria o quaternaria delle proteine stesse, causa una perdita di efficienza o sicurezza del farmaco. [1][2]

La stabilità della proteina è quindi un aspetto cruciale, indi largamente investigato, nello sviluppo delle formulazioni. Stabilità della proteina si traduce nella tendenza a mantenere la struttura nativa o *folded,* che è biologicamente attiva. Mentre una molecola proteica che perda la propria conformazione originaria, quindi si trovi nello stato *unfolded*, generalmente non è biologicamente attiva. Non è pertanto in grado di espletare alcuna funzione terapeutica. [3]
Le cause di instabilità si possono ricondurre a due macro-categorie: instabilità di tipo chimico e instabilità di tipo fisico. Il seguente studio si focalizza sulla seconda categoria, alla quale sono afferenti fenomeni quali, tra gli altri, denaturazione e aggregazione. [4] [5][6][7]

La denaturazione consiste nella perdita della conformazione stabile della proteina, a seguito di modificazioni nella struttura secondaria o terziaria. Può essere indotta da differenti processi:

- Denaturazione chimica, dovuta all'aggiunta di agenti denaturanti (e.g. urea, cloruro di guanidinio);
- Denaturazione termica, a seguito dell'aumento di temperatura, la quale consente di ricavare un parametro detto *melting temperature* T_m, che indica la propensione della proteina all'*unfolding*.
- Denaturazione a freddo, che si verifica a basse temperature e consiste nell'esposizione dei gruppi idrofobici, generalmente concentrati nel cuore della struttura, all'acqua contenuta nella soluzione
- Denaturazione indotta dalla pressione, ad altissime pressioni la proteina tende a passare allo stato *unfolded* poiché il volume occupato è minore.

L'aggregazione è uno dei fenomeni degradativi più frequenti, poiché può verificarsi durante qualsiasi fase della preparazione, del trasporto o dello stoccaggio. Molti studi correlano l'aggregazione e l'insorgenza di malattie neurodegenerative, mentre, per quanto riguarda l'aspetto terapeutico, l'aggregazione causa immunogenicità. [5][6][7]
Può essere indotta da stress fisico della proteina, dalle variazioni di forza ionica della soluzione o dall'occorrenza di reazioni chimiche che denaturano in parte la proteina. Essa è il risultato di una carenza in stabilità conformazionale e in stabilità colloidale della proteina in soluzione; la prima quantificata da ΔG_{unf}, la seconda dal coefficiente viriale B_{22}. [7]

L'*unfolding* totale o parziale della proteina si può verificare all' interfaccia liquido-aria, liquido ghiaccio o liquido-solido (adsorbimento sulla superficie del contenitore.

Questo fenomeno è dovuto alla natura anfifilica delle proteine, le quali solitamente presentano residui idrofobici nel cuore della struttura e residui idrofili esposti al solvente. In presenza di interfacce, esse tendono ad accumularvisi, poiché trovano un ambiente favorevole per entrambi i tipi di residui. [5] La più critica è l'interfaccia acqua-aria, in quanto pressocché inevitabile. [8] Inoltre, può verificarsi la *freezing-induced denaturation*, correlata alla denaturazione a freddo ma anche alla cristallizzazione dell'acqua, che porta alla formazione di interfaccia acqua-ghiaccio e all'aggregazione. [9]

Per aumentare la stabilità della proteina è necessario prestare numerosi accorgimenti durante la preparazione. In primis è opportuno scegliere il buffer adatto, poiché il pH è un fattore influente. Bisogna anche valutare l'effetto dell'aggiunta di sali, che influenzano la stabilità colloidale, per interazione diretta, effetto schermante di carica, variazioni di forza ionica. [5]

Una delle tecniche più adoperate per incrementare la stabilità dei biofarmaci a base di proteine è la liofilizzazione, che si realizza in tre step: congelamento, essiccamento primario a bassa pressione ed essiccamento secondario in condizioni di vuoto spinto. La potenza di questa tecnica risiede nella rimozione dell'umidità che causa degradazione di tipo fisico-chimico. Tuttavia, durante la liofilizzazione, la proteina può essere danneggiata secondo numerosi meccanismi: pH shift, concentrazione della proteina, causata da cristallizzazione del solvente e generante aggregazione, denaturazione a freddo, *unfolding* all'interfaccia acqua-ghiaccio. [10][11]

Altro fattore rilevante nell'incremento della stabilità è la scelta degli eccipienti da aggiungere alla formulazione. Essi sono ripartiti in due classi:

- Osmoliti (zuccheri, polioli, amminoacidi, sali), incrementano la stabilità della proteina grazie ad interazioni di tipo repulsivo. In soluzione, tramite un fenomeno detto "esclusione preferenziale", l'eccipiente si concentra maggiormente nel bulk rispetto alla zona prossima alla superficie proteica e, essendo l'interazione proteina-eccipiente termodinamicamente sfavorita, la proteina tende a mantenere la struttura nativa, esponendo meno superficie possibile al solvente ricco in eccipiente. Gli osmoliti proteggono la proteina anche durante la liofilizzazione tramite i meccanismi di "water replacement" o "vitrification". [12]
- Surfattanti, proteine, polimeri, limitano l'aggregazione legandosi debolmente alla superficie della proteina ("complesso surfattante-proteina") o interagendo con le interfacce, competendo contro la proteina stessa ("competizione interfacciale"). [13]

Una nuova classe di eccipienti sono le ciclodestrine, una famiglia di oligosaccaridi ciclici formati da unità di glucopiranosio. Le classi di ciclodestrine più largamente impiegate e studiate sono:

- α-CDs formate da sei unità
- β-CDs formate da sette unità
- γ-CDs formate da otto unità

Esse presentano struttura tronco-conica cava, la cui parte esteriore è idrofila mentre quella interiore (la cavità) è idrofoba. [14] [15] La presenza di tale cavità consente alle CDs di incapsulare molecole o formare complessi ("inclusione preferenziale") con le molecole ospiti. La complessazione è stata rilevata in particolare per la β-CD, che è anche la meno costosa e più largamente disponibile. Tuttavia, tale molecola è anche la meno solubile tra le CDs native e quindi quella con più pronunciata azione emolitica. [15] [16]

Per ridurre il potenziale tossico della β-CD è stata funzionalizzata. La più utilizzata tra le derivatizzate è la idrossipropil-β-ciclodestrina, HP-β-CD, che è infatti tra le CDs investigate in questa trattazione.

Le β-CDs sono state oggetto di numerosi studi negli ultimi anni e si sono dimostrate efficienti nell'inibire l'aggregazione e, specialmente la HP-β-CD, nell'agire come lioprotettore anche per la LDH, una delle due proteine su cui l'attività di ricerca per questa trattazione si è focalizzata.

Le proteine investigate sono appunto:

- Granulocyte colony stimulating factor (GCSF), una glicoproteina che stimola la produzione e il rilascio nel sangue di granulociti neutrofili e cellule staminali, risultata efficace nel trattare la neutropenia, il morbo di Parkinson e danni al miocardio. Ha un peso molecolare di 18110 Da e dimensioni 45 Å x 26 Å x 26 Å. [17][18][19]
- Lactate Dehydrogenase (LDH), enzima tetramerico contenuto in quasi ogni tessuto del corpo umano. Catalizza la conversione di lattato in piruvato, riducendo la nicotinammide adenina dinucleotide (NAD) a NADH. È impiegata come indicatore del danno cellulare e nella produzione di solventi biodegradabili. La struttura include il 40% di α-eliche e il 23% di β-foglietto. Il raggio idrodinamico è circa 4.2 mm. [20][21][22]

L'obiettivo di questo lavoro è lo studio delle ciclodestrine come eccipienti nelle formulazioni a base di proteine. È stato investigato il comportamento di GCSF e LDH in formulazioni contenenti β-CD, HP- β-CD e maltoeptaosio, corrispondente alla forma a catena aperta della β-CD, per distinguere i tratti attribuibili alla forma caratteristica delle CDs.

Il piano sperimentale è stato elaborato in modo da testare la stabilità delle proteine in presenza e in assenza dei suddetti eccipienti, valutando la capacitò delle CDs e preservare struttura e attività delle proteine.

La sperimentazione è stata effettuata presso i Laboratori della Facoltà di Biofarmacia presso la Ludwig Maximilian Universität (LMU) di Monaco di Baviera, in collaborazione con il Dipartimento di Ingegneria chimica e dei materiali del Politecnico di Torino.

I risultati della sperimentazione sono stati appunto comparati con quelli delle simulazioni di Dinamica Molecolare, effettuate presso la struttura torinese.

Materiali e metodi

La GCSF è stata formulata in soluzione 10 mM di acetato di sodio a pH 4.5, la concentrazione di proteina, a meno di differenti precisazioni, è stata fissata a 1 mg/mL e la concentrazione dei tre eccipienti, β-CD, HP-β-CD e maltoeptaosio, è di 15 mM.

La LDH è stata formulata in solizione 10 mM di fosfato di potassio a pH 7.0, la concentrazione di proteina è stata fissata a 0,5 mg/mL e la concentrazione di eccipienti è di 10 mM. Per la seconda parte della sperimentazione sull'attività della proteina la concentrazione di LDH è stata invece fissata a 0,005 mg/mL. Nel contesto del saggio di attività, la LDH è stata formulata anche con saccarosio e con cloruro di sodio, oltre che con i tre eccipienti sopracitati.

I test accelerati di stabilità a cui la proteina è stata sottoposta, per simulare in modo più spinto gli stress a cui è sottoposta durante la preparazione, il trasporto e la conservazione, sono i seguenti:

- Agitazione: 1400 rpm per 1 h e per 5 h;
- Congelamento e scongelamento: singolo ciclo e 5 cicli consecutivi
- Liofilizzazione (steps, con relative pressioni e temperature, in **Table 3**)
- Conservazione di campioni liquidi appena preparati a 4°C per 14 giorni e 28 giorni, a 40°C per 14 giorni e 28 giorni e conservazione di campioni liofilizzati a 40°C per 28 giorni.

Le tecniche analitiche utilizzate per indagare il comportamento della proteina prima e dopo le sollecitazioni a cui è stata sottoposta consentono di concentrarsi su tre aspetti principali:

1. <u>Formazione di legame tra la proteina e l'eccipiente</u>
   - Biolayer Interferometry (BLI), in cui la proteina è immobilizzata su un sensore, che viene immerso nella soluzione contenente l'eccipiente e si valuta se lo spessore dello strato sul sensore ne risulta modificato, a causa del legame dell'eccipiente alla proteina.
   - Microscale Thermophoresis (MST), in cui si valuta il movimento delle molecole sottoposte a un gradiente di temperatura, misurando la fluorescenza, il cui valore dipende anche dalle dimensioni della molecola e ci consente di distinguere tra il caso in cui l'eccipiente è legato alla molecola e il caso in cui non lo è.
2. <u>Aggregazione e stabilità conformazionale della proteina</u>
   - Misura del pH
   - Dicroismo circolare, in cui si tiene conto, tramite l'ellitticità, delle differenze nell'assorbimento della luce polarizzata circolare destra e polarizzata circolare sinistra; l'assorbimento viene valutato nella regione far-UV, per investigare la struttura secondaria (presenza di α-eliche e β-

foglietto), e nella regione near-UV per ottenere informazioni sulla struttura terziaria grazie ai residui aromatici.
- Differential Scanning Fluorimetry (NanoDSF), utilizzata per investigare la stabilità termodinamica della proteina, si applica un gradiente di temperatura e si misura il cambio in fluorescenza intrinseca della molecola; consente di individuare la T_m (temperatura alla quale la proteina è denaturata al 50%) e la T_{agg} (temperatura alla quale si rileva il primo aggregato).
- Denaturazione chimica isotermica, effettuata con urea, grazie alle misure di fluorescenza sono stati individuati ΔG_{unf} e C_m (concentrazione di urea necessaria a denaturare la proteina)
- Dynamic Light Scattering (DLS), in cui si valuta la diffusione delle molecole in soluzione quando vengono percorse da un fascio di luce; essendo tale comportamento dipendente dalla dimensione delle molecole, è possibile ricavare il raggio idrodinamico e la polidispersità percentuale, oltre che il parametro di interazione kD, dalla pendenza della retta che esprime la dipendenza del coefficiente di diffusione dalla concentrazione, esso consente di determinare la presenza di interazioni attrattive o repulsive.
- Fluid Imaging Microscopy (FlowCam), consente di identificare le particelle in campioni liquidi, classificandole in base alla dimensione.
- Torbidità, misurata solo per i buffer in presenza di β-CD.

3. <u>Attività delle proteine</u>
   - Reverse-phase High-Performance Liquid Chromatography, utilizzata solo per la GCSF, per distinguere il monomero della proteina da altre specie maggiormente idrofile o idrofobe, formatesi a seguito delle sollecitazioni, sulla base delle differenze nei tempi di eluizione.
   - Saggio di attività calorimetrico, utilizzato per la LDH, monitora l'attività della proteina guardando all'incremento di assorbanza all'avanzare della reazione di riduzione da NAD a NADH.

Risultati e discussione

GCSF

La formazione del legame tra la proteina e gli eccipienti, investigata tramite BLI, ha fornito un debole segnale per la β-CD ma il valore dell'intensità ha mostrato una leggera dipendenza dalla concentrazione (fig.8). Il segnale rilevato è invece più chiaro e intenso per la HP-β-CD e anche in questo caso si può riconoscere una dipendenza dalla concentrazione ma, avendo uno dei campioni mostrato tendenza inversa, è stato necessario ricercare la conferma nell'applicazione dell'altra tecnica analitica (fig.9).
Il maltoeptaosio, incluso come *negative control*, ha mostrato un segnale intenso, seppur di forma non coerente con la crescita del biolayer a seguito del legame.

I risultati del BLI trovano completa conferma in quelli forniti dalla MST. Infatti, la fluorescenza relativa cresce con la concentrazione di eccipiente nel caso della HP-β-CD (fig.13), indicando che la formazione di legame è favorita all'aumentare della concentrazione, poiché è maggiore la probabilità che eccipiente e molecola

interagiscano. Mentre il legame tra β-CD e GCSF è stato rilevato soltanto per la massima concentrazione di eccipiente, 10 mM (fig.14). Ancora una volta per il maltoeptaosio il segnale di fluorescenza relativa è spiccato ma non è distinguibile una concentrazione per cui l'eccipiente si possa dire effettivamente legato alla proteina (fig.15).

Questi risultati sono in controtendenza rispetto a quanto atteso dalle simulazioni che invece suggerivano una più spiccata propensione all'inclusione preferenziale da parte della β-CD. [23]

A seguito di questa valutazione preliminare è stato dapprima misurato il pH, le cui variazioni possono promuovere l'aggregazione. È stato rilevato un aumento di pH dopo la liofilizzazione, eccetto nel caso in cui la proteina è stata formulata con HP-β-CD. Nel caso di β-CD, l'aumento di pH verso valori più favorevoli all'aggregazione è stato meno pronunciato che in assenza di eccipienti ma comunque presente.

Dallo spettro ricavato tramite dicroismo circolare nella regione near-UV, la struttura terziaria della GCSF non risulta modificata dall'aggiunta degli eccipienti alla formulazione. Per quanto riguarda la struttura secondaria, permangono i minimi nello spettro far-UV in corrispondenza di 222 e 208 nm, che denotano la presenza di α-eliche ma i valori di ellitticità non sono conformi a quelli attesi da letteratura e richiedono ulteriori analisi (fig 16 e 17).

Per quanto riguarda la denaturazione termica e quella chimica, solo la HP-β-CD mostra effetti positivi, in quanto il campione formulato con il suddetto eccipiente mostra leggero incremento rispetto alla GCSF in assenza di eccipienti nei valori di T_m, T_{agg} (tab. 3) e C_m (fig. 22).

La differenza in T_{agg} non è stata invece rilevata adoperando la tecnica del DLS, tuttavia tale tecnica ha mostrato dei limiti nell'investigazione di GCSF formulato con CDs. Ha consentito infatti di rilevare la presenza di forze attrattive, in quanto i valori di k_D sono negativi, ma il valore del coefficiente di diffusione a diluizione infinita D_0 (tab.6), che dovrebbe essere uguale in tutte le formulazioni, è più elevato in presenza di CDs. Ciò significa che le CDs danno un segnale, poiché diffondono più velocemente della proteina.
Tale assunzione è confermata anche dalle distribuzioni di dimensioni ottenute grazie alla medesima tecnica, le quali mostrano un allargamento del picco in corrispondenza di un raggio di 2 nm (monomero) verso valori più vicini al raggio delle CDs, che si attesta su circa 0,8 nm (fig. 25-30).

La distribuzione di dimensioni è stata indagata più nel dettaglio prima e dopo ogni stress sulla proteina, tramite Fluid Imaging Microscopy. È stata rilevata una presenza massiva di particelle di diametro inferiore a 1 μm (fig. 32), nelle formulazioni contenenti β-CD, anche prima che qualsiasi sollecitazione fosse applicata. Ciò è riconducibile alla scarsa solubilità della β-CD (15 mM è molto prossimo al limite di solubilità che si attesta attorno ai 16 mM). Infatti, la presenza di particelle è sensibilmente ridotta dopo conservazione a 40 °C, mentre diventa più importante quando il campione viene portato a basse temperature. Il maltoeptaosio mostra la

stessa tendenza, seppur meno spiccata. Queste due sostanze hanno mostrato anche una tendenza a promuovere l'aggregazione (fig. 33). La GCSF formulata con HP-β-CD mostra comportamento analogo alla formulazione priva di eccipienti, anzi si mostra efficiente nel prevenire l'aggregazione nel caso della liofilizzazione. Inoltre, la HP-β-CD e il maltoeptaosio sono le uniche sostanze tra quelle testate a prevenire il collasso delle cake liofilizzate. Non è altrettanto efficiente se i campioni liofilizzati vengono poi conservati a lungo a 40 °C, anzi in questo gruppo di campioni si registra il maggior numero di aggregati, qualunque sia l'eccipiente, poiché è probabile che la naturale propensione della GCSF di aggregare a 37 °C, sia enfatizzata dalla formazione dell'interfaccia acqua-ghiaccio e dalla disidratazione, avvenute durante la liofilizzazione.

È stata infine valutata la capacità della GCSF di preservare la propria attività a seguito delle varie sollecitazioni. Lo si è fatto in termine di *monomer recovery* (fig.34), comparando appunto la quantità di monomero residua dopo ogni stress, rispetto a quella misurata nei campioni appena preparati. I valori mostrano recupero pressocché totale in ogni caso, eccetto dopo la liofilizzazione e soprattutto la conservazione a temperature elevate. È ragionevole credere che la proteina persa abbia formato aggregati e sia stata rimossa nella centrifugazione che precede questo tipo di misurazioni. È anche opportuno evidenziare che, in presenza di HP-β-CD si rilevano picchi corrispondenti a specie idrofile, sia dopo i periodi di conservazione a basse che ad alte temperature e anche nel caso di liofilizzazione e di successivo stoccaggio dei liofilizzati a 40 °C. questo potrebbe indicare l'occorrenza di fenomeni di degradazione chimica come l'ossidazione.

LDH

Non è stato possibile investigare direttamente la formazione del legame tra proteina ed eccipienti poiché la LDH non si lega efficacemente al sensore adoperato nel BLI.

Sono state invece largamente testate la stabilità conformazionale e l'occorrenza di aggregazione.

La struttura terziaria risulta leggermente modificata dall'aggiunta di eccipienti ma non è possibile ricondurre tale aspetto alla particolare forma tronco-conica delle CDs, in quanto non è apprezzabile differenza tra la forma chiusa della β-CD, quella aperta e quella idrossipropilata (fig. 41). Tuttavia, gli eccipienti non sembrano conferire maggiore stabilità termodinamica alla proteina.

Anche per la LDH le distribuzioni dimensionali delle particelle, ricavate tramite DLS, sono influenzate dalla presenza delle CDs (fig. 44-49) ma in questo caso i campioni non risultano polidispersi, come accade invece per la GCSF, nonostante PD% presenti un aumento dovuto al segnale dato dalle suddette

Il raggio idrodinamico misurato si attesta sempre sui 4 nm (fig.50), ovvero quello della proteina, eccetto nei campioni liquidi e liofilizzati conservati a 40 °C per 28 giorni. Dall'indice di polidispersità si deduce anche che questi campioni presentano una distribuzione di dimensioni molto varia (fig. 51).

I risultati ottenuti tramite Fluid Imaging Microscopy evidenziano numero contenuto di particelle e comparabile con quello rilevato subito dopo la preparazione, nel caso di shaking per un'ora, singolo ciclo di congelamento e scongelamento e di conservazione a 4°C o a 40°C. L'ammontare di particelle risulta invece più elevato negli altri casi, in particolare nei campioni contenenti β-CD, la cui solubilità è maggiore a pH 7.0 ma comunque ridotta al diminuire della temperatura. Non sono apprezzabili differenze significative tra i campioni contenenti HP-β-CD e quelli privi di eccipienti. È importante sottolineare, inoltre, che nei campioni liquidi conservati a 40°C si è riscontrata la formazione di un sottile strato trasparente, probabilmente formato da proteina aggregata, e che sia quindi riconducibile a questo il basso numero di particelle rilevate (fig. 52 e 53).

L'attività della LDH è stata monitorata grazie ad un saggio calorimetrico. Quando formulata con HP-β-CD, la proteina presenta valori maggiori di attività, eccetto nei campioni conservati a 40 °C. L'attività risulta circa raddoppiata quando i campioni sono sottoposti a congelamento-scongelamento, liofilizzazione o conservazione prolungata a 4°C. Dal momento che tale risultato è inverso a quanto atteso, l'attività dei campioni sottoposti a cicli di congelamento-scongelamento è stata misurata nuovamente dopo averli mantenuti per 3 giorni a 4°C ed è risultata più bassa (fig. 57). Per quanto concerne la HP-β-CD, la sua attività di crioprotettore e lioprotettore è attestata in letteratura. Per quanto riguarda il risultato nella sua globalità, pur essendo in controtendenza rispetto alle aspettative, essendo le condizioni ottimali di temperatura per LDH da muscolo di coniglio attorno ai 50 °C, simili esperienze sono documentate in letteratura. Dal momento che la LDH può esistere in differenti conformazioni attive, si può ipotizzare che stress non-denaturanti promuovano il passaggio a conformomeri che presentano valori di attività più elevata. Inoltre, si potrebbe ricondurre questo comportamento ad un meccanismo di auto-protezione messo in atto dalla proteina e promosso alla concentrazione testata.

In ragione di quanto detto, si sono ripetute tutte le misurazioni dopo aver sottoposto a situazioni di stress per la proteina dei campioni in cui la concentrazione della suddetta era 100 volte inferiore. Sono stati aggiunti al set anche campioni in cui la proteina era formulata con saccarosio o con cloruro di sodio, il primo un crioprotettore largamente documentato, il secondo un agente caotropico, promotore di denaturazione. Anche in questo caso l'attività del campione contenente HP-β-CD è più alta già in partenza. Lo shaking produce una perdita di attività, eccetto laddove è presente HP-β-CD, che si accumula all'interfaccia acqua-aria, impedendovi l'accesso alla proteina. Per quanto riguarda l'aumento di attività, a seguito di liofilizzazione o congelamento-scongelamento, è meno spiccato rispetto ai campioni più concentrati ma il trend è il medesimo. Anche in presenza di saccarosio si verifica ma in modo meno pronunciato rispetto al caso in presenza di HP-β-CD, in accordo con la letteratura che lo vede meno efficace rispetto alla CD nel ruolo di lioprotettore per la LDH. Il cloruro di sodio non ha causato un calo di attività forte quanto atteso ma probabilmente soltanto perché la sua concentrazione non era sufficientemente elevata da indurre denaturazione completa (fig.58).

β-CD

Per confermare le ipotesi legate alla solubilità, sulla presenza importante di particelle nei campioni contenenti β-CD, sono stati sottoposti a Fluid Imaging Microscopy i buffer, in assenza di proteina. In buffer acetato, a pH 4.5 è stata rilevato un numero di particelle molto elevato in presenza di β-CD e maltoeptaosio, mentre in presenza di HP-β-CD o in assenza di eccipienti il numero di particelle è a confronto irrisorio.

Tale considerazione vale sia che si consideri la classe di particelle con diametro maggiore di 1 μm che quella di particelle di diametro maggiore di 10 μm (fig.61 e 62).

Lo stesso si può dire se si considera buffer fosfato a pH 7.0, ma l'ammontare di particelle, in termini assoluti, è inferiore (fig.63 e 64). Ciò evidenzia l'importanza del pH nella preparazione di biofarmaci a base di proteine.

Per decidere se la β-CD possa essere studiata a concentrazioni minori, in cui il problema della solubilità venga meno, è stata utilizzata Fluid Imaging Microscopy ed è stata valutata la torbidità nei due buffer contenenti concentrazioni differenti di β-CD. Da tali valutazioni emerge che, per evitare il problema della solubilità, a pH 4.5 non si può avere una concentrazione di eccipiente superiore a 5 mM, mentre a pH 7.0 il problema si presenta dal valore di 15 mM in poi (fig 65-68).

Conclusioni

Questo studio ha investigato la possibilità di impiegare le ciclodestrine come eccipienti e ha messo a confronto la β-CD, la meno costosa e più largamente disponibile tra le CDs, con la sua forma idrossipropilata e con la forma aperta. La derivatizzazione ha il vantaggio di incrementare la solubilità, riducendo il potenziale tossicologico, mentre la forma aperta ci consente di mettere in luce gli aspetti effettivamente legati alla particolare struttura tronco-conica delle CDs.

Dallo studio sulla GCSF, emergono i limiti legati alla solubilità della β-CD, che risulta inadatta ad essere utilizzata come eccipiente a pH 4.5 e/o a basse temperature, in quanto incrementa il numero di particelle e favorisce la formazione di aggregati. Tale propensione non era attesa dai risultati delle simulazioni di dinamica molecolare, che invece prospettavano tendenza più spiccata alla formazione di clusters in presenza della forma idrossipropilata.

La HP-β-CD si è rivelata la più efficace contro denaturazione termica e chimica, limita lo shift di pH durante la liofilizzazione e inibisce la formazione di aggregati durante i cicli di congelamento-scongelamento, in cui il farmaco può incorrere durante trasporto o stoccaggio, e durante la liofilizzazione, che è una delle tecniche più largamente adottate per prolungare la shelf life dei biofarmaci.

Entrambe le CDs si sono mostrate valide nel preservare l'attività della proteina, tuttavia nel caso della HP-β-CD, sarebbe necessario attuare ulteriori verifiche sull'occorrenza di percorsi degradativi quali l'ossidazione. Questo rimane comunque il più promettente tra gli eccipienti studiati.

Tale studio ha anche evidenziato i limiti della tecnica del Dynamic Light Scattering, poiché le CDs diffondono più velocemente della proteina impedendo valutazioni corrette sui parametri chiave per valutare la stabilità colloidale.

Dallo studio sulla LDH è emerso la capacità della HP-β-CD di agire come surfattante, impedendo una denaturazione della proteina all'interfaccia acqua-aria ed a quella acqua-ghiaccio ed una conseguente perdita di attività. Inoltre, è stata riscontrato un particolare incremento di attività quando la proteina è formulata con la HP-β-CD, in particolare a seguito di cicli di congelamento e scongelamento o di liofilizzazione, probabilmente riconducibile ad un cambio di conformazione della proteina. Tale aspetto andrebbe ulteriormente investigato.

Tale lavoro ha evidenziato la necessità di effettuare sempre verifiche sperimentali a valle di simulazioni di Dinamica Molecolare, che restano comunque uno strumento molto potente per investigare i meccanismi di interazione. Questo studio si colloca inoltre nel crescente filone di ricerche che si interessa alle ciclodestrine e in particolare ha messo in luce le grandi potenzialità della HP-β-CD.

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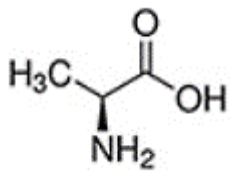
Chapter 1

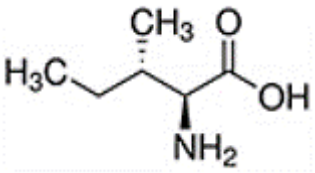
Introduction

Proteins are natural molecules that perform important functions in our body and their role is the maintenance of good health. In the last decades, biopharmaceutics have developed in order to detect and treat diseases that arose from malfunctioning or absence of proteins.
They are large biomolecules which consists of one or more long chains of amino acid residues which are listed below:

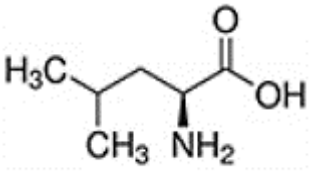
- Amino acids with hydrophobic side chain – Aliphatic



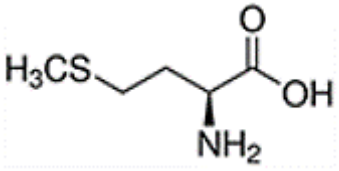
Alanine, **Ala**



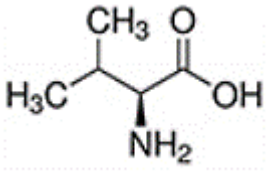
Isoleucine, **Ile**



Leucine, **Leu**

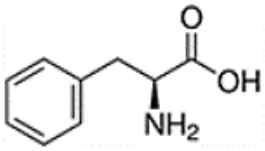


Methionine, **Met**

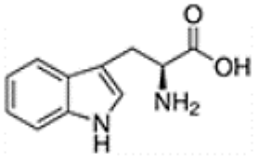


Valine, **Val**

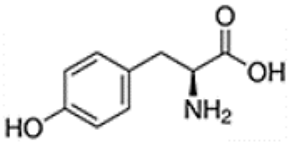
- Amino acids with Hydrophobic side chains – Aromatic



Phenylalanine, **Phe**

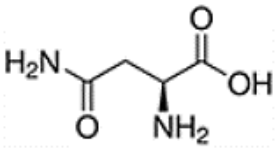


Tryptophan, **Trp**

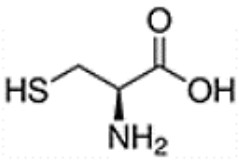


Tryosine, **Tyr**

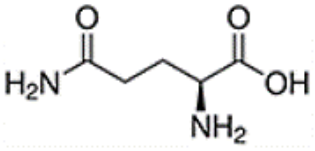
- Amino acids with polar neutral side chains



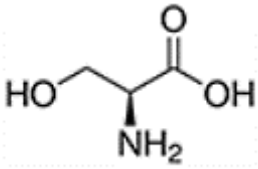
Asparagine, **Asn**



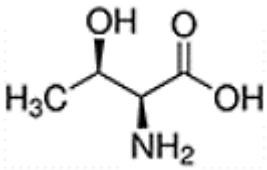
Cysteine, **Cys**



Glutamine, **Gln**

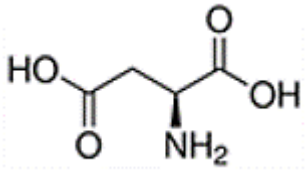


Serine, **Ser**

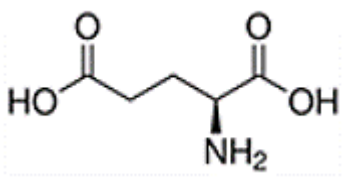


Threonine, **Thr**

- Amino acids with electrically charged side chains – Acidic

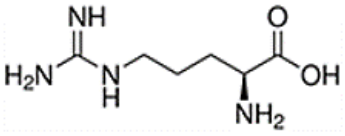


Aspartic acid, **Asp**

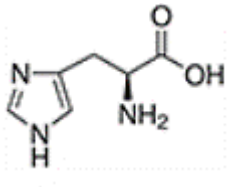


Glutamic acid, **Glu**

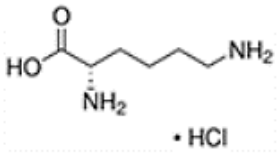
- Amino acids with electrically charged side chains – Basic



Arginine, **Arg**

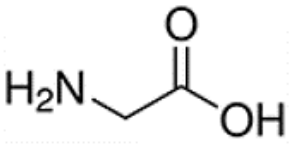


Histidine, **His**

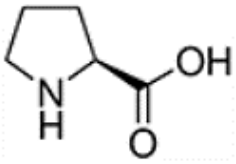


Lysine, **Lys**

- Unique amino acids



Glycine, **Gly**



Proline, **Pro**

Proteins are a growing class of therapeutic agents investigated in the biopharmaceutical field. Because of the large, complex structure, chemical and biological differences, proteins for pharmaceutical application must be, characterized, manufactured, and delivered in different ways respect than the traditional small molecules drugs. Modifications in proteins primary, secondary, tertiary or quaternary structure can affect efficiency and safety of the drug products. [1][2]

Loss of native structure may occur at different stages of the pharmaceutical product life; thus, the protein stability is a crucial topic in development of formulations. This chapter will give a concise excursus through causes of proteins instability, methods used to improve stability, and substances on which the research focuses.

1. Proteins instabilities

The stability of proteins is the tendency to maintain the folded state, also called "native conformation". This conformation is biologically active, while the unfolded one is usually biologically inactive. In physiological conditions, for typical proteins, the change in Gibbs free energy is about 20-65 kJ/mol. The unfolded state is favored by high degree of conformational entropy, while the folded state is maintained by strong peptide bonds and weak interactions like hydrogen bonds, Van der Waals forces, hydrophobic and ionic interactions. [3]
The stability of formulations containing proteins is a crucial research topic. The instability pathways are divided into two classes: chemical instability and physical instability.
The former involves formation or breaking of covalent bonds, which lead to formation of new chemical entities. The latter implies changes in the physical state of the protein, while the chemical composition is not affected by processes like denaturation, aggregation, precipitation and adsorption. [5]

Chemical instability can occur in several ways. The most common chemical degradation pathway is deamidation that consists in the hydrolysis of Asn and Gln side chain amides; it generates process-related impurities and degradation product, and some studies revealed its contribute to increase immunogenicity. [5],[24]

Other pathways that may occur are Asp isomerization, Asp hydrolysis, Hinge Region hydrolysis, racemization or β-elimination of Asp residues, diketopiperazine (DKP) ring formation, N-terminal glutamate cyclization to form pyroglutamate, disulfide scrambling usually occurring at alkaline pH.

Particularly relevant are glycation and oxidation. Glycation is a reaction in which free amino groups of proteins are modified by monosaccharides; it is induced by reducing sugars in long term storage. [25] Oxidation is definitely an important issue, since it can potentially occur in any protein containing His, Met, Cys, Tyr and Trp amino acids, damaging the protein. Different stages of the protein purification, formulation and storage may induce oxidation of these reactive side chains. [5]

This work will focus more on physical stability issues, since they are strongly relevant in the study of cyclodextrins used as excipients, in formulations based on Lactate Dehydrogenase (LDH) and Granulocyte Colony-Stimulating Factor (GCSF).

1.1. Denaturation

The denaturation of proteins is the loss of its initial and most stable conformation, also called native state. The partial or total unfolding, which implies the change in the secondary or tertiary structure of the protein, leads the protein to adopt a new conformation named “denatured state”. The stability is a critical property for protein function and for preventing the accumulation of cytotoxic unfolded or misfolded protein forms. [4] There are many ways to induce denaturation of proteins and are discussed below.

Chemical denaturation is based on the addition of denaturing agents (the most common are urea and guanidinium hydrochloride) to the solvent. Urea forms hydrogen bonds with peptide backbone, disrupting the native form; guanidinium hydrochloride disturbs hydrophobic interactions of the native structure, in particular those involving aromatic side chains. Even if the polypeptide chain remains chemically intact, the biological function of the protein is lost. The chemical denaturation is commonly used to determine the stability of globular proteins, measuring the free energy of unfolding. [5][26][27]

Thermal denaturation is induced by temperature increase, which causes the partial or total unfolding of the protein, breaking the forces between charged groups and forces of mutual attraction of nonpolar groups.[28] The solution containing the protein in its native state is heated up, until the complete denaturation of the protein is reached. The temperature in which native and denatured state are equally present (50% of molecules unfolded, the remaining 50% in the native state) at equilibrium is called melting temperature (T_m). This temperature gives an information about the stability of the protein; more specifically it is the temperature of heat denaturation with $\Delta G(T_m) = 0$ and implies a change in the optical properties of the solution. For many proteins the unfolding temperature is in the range of 40-80 °C. [4][29] In formulation (e.g. buffer, pH, excipient) development thermal denaturation is employed in the research of conditions that would increase T_m.

Despite the fact that low temperature should promote the most ordered structure, the native state, the low temperature enhances the interaction between nonpolar groups of the protein and water. This phenomenon is known as cold denaturation. This is due to the fact that at low temperature the Gibbs free energy of hydration is negative and decreases with temperature. Thus, at a sufficiently low temperature, the affinity between the nonpolar groups and water increases and the polypeptide chain tends to unfold to expose these groups to water. [30]

The denaturation could also be induced by the application of high hydrostatic pressure. This phenomenon implies that the molar volume of the unfolded state is smaller than the volume of the folded state. The kinetics of the unfolding is slower than that of thermal and chemical denaturation. [5][27]

1.2. Aggregation

The aggregation of protein in pharmaceutical formulations makes the protein itself less bioactive, commercially unavailable, because of the solution appearance which is turbid or physically separated from aggregates, and is related to immunogenicity effects during therapy.[5]

Aggregation is considered one of the most dangerous degradation pathways, since it can occur in many stages of production, transportation and storage of the protein drug, e.g., shearing/shaking, freeze-thawing, freeze-drying, reconstitution. The formation of soluble/insoluble aggregates could be due either to simple physical association of molecules or to the formation of covalent bonds. The aggregation occurs mostly in presence of unfolding intermediates. The aggregation is usually physical, but it could also be due to formation or exchange of disulfide bonds, oxidation, and the Maillard reactions. [31]

The aggregation process has been widely investigated for GCSF, one of the two proteins on which this study is focusing. Aggregation can occur even if the concentration of the protein is below the solubility limit at the given temperature and under conditions in which the folded state is thermodynamically more stable than the unfolded one, because the process of aggregation is driven by intermolecular forces. The osmotic second virial coefficient B_{22} provides a measure of the interaction between spherical bodies in solution, so it is useful to predict the behavior of molecules in non-ideal solutions: positive values of B_{22} correspond to the dominance of repulsive forces between protein molecules, while negative values reflect the presence of attractive forces, which favor the aggregation. This coefficient considers the colloidal stability, but it is also necessary to take into account the conformational stability, which is quantified through ΔG_{unf}. The aggregation is associated to changes in secondary and tertiary structure of the protein, in particular the increase in β-sheet content, replacing native α-helix. Thus, the aggregation is a combination of a lack in conformational stability and in colloidal stability of the protein in solution.[7]

1.3. Proteins at interfaces

The partial or total unfolding of the proteins may occur at interfaces, for example the air-liquid interface, related to spray- drying and the ice-liquid interface in the freeze-drying.
Proteins are large, amphipathic molecules; thus, they tend to accumulate at the interfaces. Concerning the fluid-solid interfaces, adsorption to surfaces is mostly irreversible, even if the interactions are non-covalent (H-bonding, electrostatic and hydrophobic interactions) and the rate, the extent and the mechanism of the process depend indeed on hydrophobicity of the surface: the more hydrophobic the surface, the greater the extent of the absorption. This is true in particular for partially unfolded molecules, since they expose some hydrophobic residues, which are usually buried in the core in the native state. The affinity to surfaces is larger the more unstable the molecules are. The proteins with huge colloidal stability adsorb on hydrophobic interfaces under conditions of changed interactions and on hydrophilic surfaces only

if electrostatically attracted. Proteins characterized by a lower Gibbs free energy of unfolding adsorb on hydrophilic or hydrophobic surfaces, no matter if the electrostatic forces are repulsive. Thus, another important factor is the electrical charge, but it is more difficult to recognize a general rule, because proteins contain many charged groups, some negative and some other positive and it is necessary to consider the distribution of charge on colloidal particles. Indeed, on hydrophilic surfaces maximum adsorption occurs at pH value close to the isoelectric point, where the repulsive interactions in solution are minimized. [5][32][33][34]

The water-air interface is problematic because the contact with air is almost impossible to avoid in every formulation and, for the liquid ones, the shipping and storage stages become challenging as well. Many studies have been performed to understand the behavior of proteins at air-water interface, enhancing the contact via stirring and mixing. It was showed that many globular proteins form a mono-layer at the interface, resulting in partial or total unfolding of the protein structure ergo in denaturation. [5][35] The negatively charged proteins are more adsorption-prone at the air-water interface, than the positively charged ones. This difference between these two classes of proteins is due to the Van der Waals interactions between protein molecules and interface, in particular to the contribution of the dispersion forces.[8]

Proteins are also sensitive to freezing-induced denaturation. This phenomenon is related to the cold denaturation previously discussed, but it also concerns the concentration of protein (due to the crystallization of water, which may induce aggregation) and to formation of an ice-water interface.[9] This is a critical issue because freeze-drying is often used to improve storage stability of the formulations and freeze-thawing cycles may occur during the storage of drugs.[9][36] The loss of the native secondary structure has been demonstrated for lactate dehydrogenase (LDH) in a study with infrared microscopy, but it could be strongly reduced with the addiction of proper surfactants.[37]

2. Improving protein stability

The factors affecting protein stability are pH, temperature, pressure, ionic strength, conformation of the protein itself. They either give rise or increase/decrease the degradation rate of the chemical and physical phenomena previously discussed.[5]

Improving protein stability is a combination and optimization of those factors and adoption of some strategies.

The following section gives an overview about the precautions used to enhance protein stability, in biopharmaceutical formulations, focusing on the widely employed technique of freeze-drying and on the choice of the proper excipient.

2.1. Buffers

First, it is necessary to choose the proper buffer to prevent changes in pH. However, the protein is often able to provide the majority of the buffering capacity, especially when high concentrated. Phosphate is reported as buffer which increases stability, probably because of its capacity to direct bind to the native state of proteins, although sodium phosphate buffer is reported to crystallize during lyophilization, thus, when biopharmaceutical are subjected to freeze-drying, to enhance their storage stability, citrate buffer is preferred over phosphate buffer[5][10]

2.2. Ions

The effects of addiction of salts to protein formulations are different: either due to direct interaction with the protein or due to charge screening. Moreover, the Hofmeister effect has been widely investigated, which correlate the solubility of protein to the different ionic species [5]
The addition of salt also affects the ionic strength, modifying the Debye length. Thus, it can either increase the colloidal stability or favor the aggregation of proteins. [5][38][39][40]

2.3. Drying

Drying is one of the most common ways to improve protein stability, because the moisture reduction inhibits a lot of physicochemical degradation pathways, since the water may induce mobilization of protein molecules. In the pharmaceutical field there are some common techniques: spray drying, freeze drying (lyophilization), spray freeze drying, supercritical fluids drying.

Freeze-drying, in particular, is widely used for protein drugs, to enhance their stability during shipping, storage and in case of occurring of temperature excursions. The lyophilization consists three steps:

1. Freezing: the temperature is lowered, inducing solidification of the solvent, which forms ice crystals. During this step, part of the solvent is still bounded

to the protein and a certain amount of water is retained in the glassy matrix formed by the product;
2. Primary drying: the pressure is decreased to cause ice sublimation. The heat required by sublimation is given to the product through the fluid flowing in the coil, placed in the shelf;
3. Secondary drying: The temperature increases while the pressure is further reduced, in order to induce desorption of solvent still adsorbed to the product and reach the required moisture level. [10][41][42]

A lot of mechanisms can damage the protein during freezing. A pH shift can occur, due to crystallization of buffer salts (well known for sodium phosphate). In the case of crystallizing solute, the temperature during drying must be maintained below the eutectic point, otherwise a liquid phase may be formed and, then, the low pressure could lead to boiling of this liquid content. For amorphous solutes the maximum temperature is the glass transition one, to avoid the collapse of lyophilized cakes. [5][10][11] The value of the maximum allowed temperature also depends on the residual moisture, because water acts as plasticizer. Thus residual moisture is essential as well, because the water content may lead to physicochemical degradation and it can also cause cake collapse, which makes the visual appearance of the product unattractive and affects the activity of the protein. [10][11]
Furthermore, freeze concentration can be observed, as well; it represents a serious issue because it may cause protein aggregation.

During freezing, the protein gets in contact with ice-water interface, tending to unfold. It is, therefore, often necessary to add surfactants to inhibit the surface-induced degradation.

Other degradation pathways investigated in freeze-dried pharmaceutical protein products are cold denaturation and dehydration that induced structural changes. Dehydration is the loss of the water shell surrounding the protein, that may lead to denaturation, because it creates an unfavorable environment for polar residues, enhancing the tendency to create hydrophobic interactions and aggregation.[43]

2.4. Excipients

The addition of excipients to formulations is a way to avoid or limit phenomena like conformational changes, denaturation, and aggregation.

Excipients can be divided into two main classes:

- Osmolytes, additives that enhance protein stability, through lack of affinity or repulsive interaction with the protein surfaces, reducing aggregation;
- Surfactants, proteins, polymers, additives that limit aggregation by either weekly binding to the protein surface or competitive adsorption to surfaces/interface, while they do not enhance protein stability.

2.4.1. Osmolytes

This macro-class of excipients includes sugars, polyols, many amino acids and salts. In solution it has been demonstrated that those excipients increase stability of protein native structure.

The use of salts in protein formulations has been throughly discussed in paragraphs 2.1 and 2.2 therefore the following treatment will focus on other classes of osmolytes.

Sugars and polyols inhibit aggregation and are able to increase thermal stability of many proteins, which showed higher T_m, when formulated with those excipients. Sucrose, threalose, sorbitol, sarcosine and glycerol are the most widely used. It has also been found that the unfolding temperature of many antibodies increases either when the polyols concentration is higher or when larger polyols are employed.

The stabilization mechanism in solution is called "*preferential exclusion*" or "*preferential hydration*". Here the major concentration of the excipient is in the bulk rather than in proximity of protein surface. This means that protein-excipient interaction is thermodynamically unfavourable and repulsion would be greater for the unfolded structure, because of the larger structure exposed; thus, the protein tends to maintain the native structure. [12]

During lyophilization the excipient must be effective against stresses induced by both freezing and drying. Sugars are used to avoid freeze-concentration.

Dehydration is generally avoided with the addition of sugars, which tend to replace the hydrogen bonds of water molecules within the protein. Disaccharides are reported to be better lyoprotectant than monosaccharides because of the higher T_g and the flexibility of the structure. [41][12] They are able to create a glassy matrix that limits mobility of protein and prevent the collapse of the lyophilized structure, allowing to obtain elegant cakes. Many amino acids are used as bulking agent, because they are able to maintain an amorphous state during freeze-drying, preventing the collapse of lyophilized cakes.[12] This lyoprotectant action has been observed also for polyols that are also called plasticizers, such as sorbitol or glycerol, which are able to lower the T_g of the protein.[5]

Protection during freezing follows the *preferential exclusion* mechanism, taking advantage of free water still present, which is gradually reduced by water crystallization.[12]

During drying the water shell surrounding protein molecules does not exist anymore and the protection mechanisms are "*water replacement*" and "*vitrification*", deeply studied for sugars.

The former consists in the hydrogen bonding between charged/polar groups of excipients and protein, once the water is removed through sublimation, in order to maintain a polar environment and preserve protein native structure. [44][45]

The latter, as mentioned earlier, is the hypothesis of the preservation of protein structure through the formation of a glassy matrix by sugars, which limits the mobility,

reducing long-order (aggregation) and short-order interactions (deamidation, cyclization, etc.).[12][46]

2.4.2 Surfactants

Surfactants are added to therapeutic protein formulations in order to avoid the interfacial stress to protein itself. The protein, being amphiphilic tends to adopt the globular structure in aqueous solution, by burying within the core the hydrophobic residues, exposing to water the hydrophilic ones. At the water-air interfaces, as previously discussed, the protein tends to unfold, to expose its hydrophobic part to air. Another important issue concerns the ice-water interface. Thus, limiting the interaction with the interfaces is a critical topic in protein formulations.[13]

Surfactants are amphiphilic molecules, consisting of a bulky polar head bonded to a hydrophobic chain. They therefore tend to accumulate at the air-water interface. or to assembly in micelles (organized structures that minimize the contact between hydrophobic part and water), up to the critical micelle concentration (CMC). The initial concentration in protein formulations development is usually near to the CMC. The proper concentration is determined with stress studies and provides an high safety margin. [13][47]

In protein formulations are widely used non-ionic surfactants, which consist in a bulky polar head bonded to a hydrophobic chain. They protect the protein against the interfacial stress and from the consequent aggregation. Moreover, the surfactants are used to prevent the precipitation in solution of the protein and to limit the interaction with cases surfaces. [13][47] Finally, surfactants are reported to be effective in assisting protein refolding. [12]

In particular, in protein formulations, are used mostly poli-oxy-ethylen baseed surfactants, e.g. Polisorbate 20 and 80 and poloxamer 188, which are performant in protecting the protein during shaking, freeze-thawing and in preventing the absorption to surfaces of filters and packages. Nevertheless the polisorbates showed some shortcomings, such as degradation via oxidation and/or hydrolysis, leading to the formation of oxygen active species, which may cause the oxydation of proteins in formulation.

The two main proposed stabilization mechanisms of proteins by non ionic surfactants are "*interfacial competition"* and "*surfactant-protein complexation"*. The former is more thermodynamically favored, according to literature and it is related to an increasing in surface tension, due to the addiction of surfactant to the solution. [13][47]

Moreover, it is important to highlight that surfactants are reported to prevent aggregation of proteins during reconstitution of lyophilized samples. This may be related either to reversibility nature of surfactant-protein interactions or to the presence of surfactant at air- solid interface that gives the time for protein refolding, retarding dissolution rate.[12]

2.4.3. Polymers

Hydrophilic polymers are widely employed as excipients, one of the most common is dextran, which has proved to be effective against heat-induced aggregation in solution and in lyophilized samples after reconstitution. Polysaccharides and inert proteins are widely employed.[12] Proteins are used in recombinant version, although the use of protein based excipients increase the formulation complexity.[33]
Polyethylene glycol (PEG), possessing a non-polar moiety, acts as surfactants and reduces water surface tension in solution, limiting aggregation phenomena.
Charged polymers stabilize proteins in solution by electrostatic interactions, e.g. polyethyleneimine (PEI) improved LDH stability storage and suppressed its aggregation, in solution and after freeze-thawing cycles. [12]
Poloxamers are demonstrated to be efficient in preventing aggregation induced by different kinds of stresses applied to proteins in solution.
Polymers like PVPs and maltodextrins are able to improve storage stability of lyophilized samples.[12]
The most common stabilization mechanism for hydrophilic macromolecules is the "molecular crowding effect: proteins tend to maintain native structure, in order to be less exposed to the polymer. This effect is enhanced when the polymer is larger. When polymers possess hydrophobic character, the stabilization is given by equilibrium between exclusion due to steric hindrance and destabilizing hydrophobic interactions. Charged polymers may also stabilize, as previously discussed through specific binding or long-range Coulombic forces.[12]

3. Cyclodextrins

Cyclodextrins (CDs) are a family of cyclic oligosaccharides, formed by glucopyranose units linked by α-(1,4) bonds. The most commons classes of cyclodextrins are:

- α-CDs formed by six units
- β- CDs formed by seven units
- γ-CDs formed by eight units

These compounds are produced via intramolecular trans-glycosylation reaction from degradation of starch by cyclodextrin glucotransferase (CGTase) enzyme. [14] Native CDs possess crystalline, non-hygroscopic, homogeneous structure. [15]

CDs present a hollow truncated-conical shape, with hydrophilic exterior and non-polar hydrophobic cavity. [14][48] The bonds between glucopyranose units do not allow their free rotation; thus the shape is not cylindrical. The primary hydroxyl groups are placed on the narrow side, while the secondary groups on the wider side. The cavity act as a cage and enable CDs to encapsulate molecules or form inclusion complexes with guest molecules. [16]

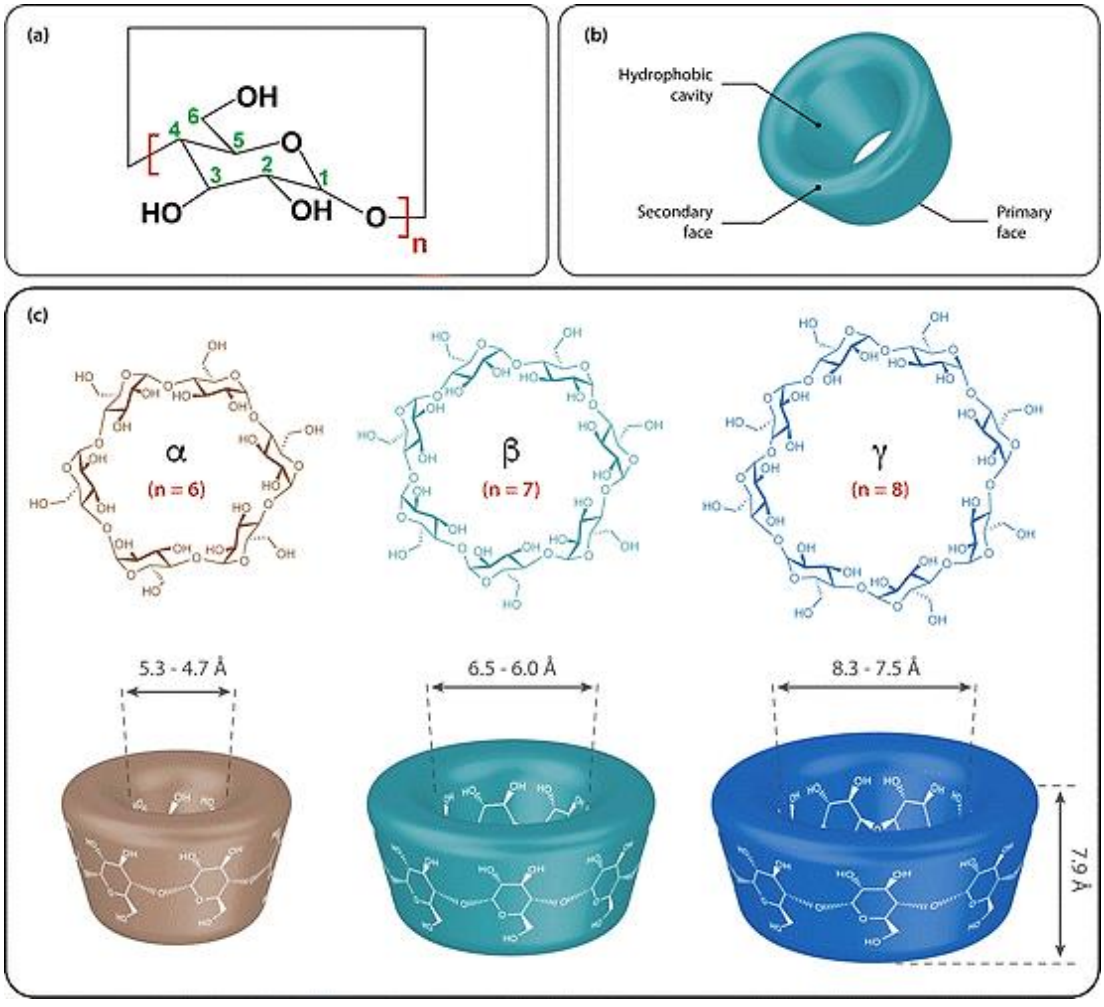


Figure 1- Glucopyranose unit (a), typical cyclodextrin structure (b) α-, β- and γ-cyclodextrins (c) [16]

This feature is particularly remarkable for β-CD, which can form inclusion complexes through host-guest interactions (Van der Waals forces, hydrophobic interactions, hydrogen bonding, etc.) with many different substances, and it is the cheapest and most easily available. The complexes can be formed by one, two or three CDs entrapping one or two molecules. CDs can also self-associate, forming micelles in solution. [14][16]

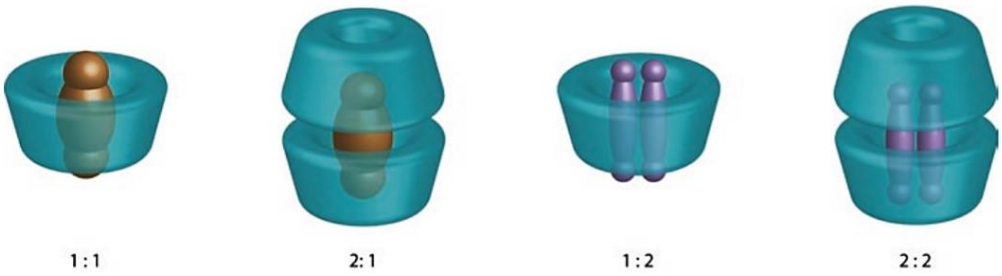


Figure 2 - Schematic representation of main CDs complexes stoichiometries [16]

From the number of scientific publications concerning cyclodextrins it is possible to notice the growing attention around their application in many different fields in the last two decades. This work focuses obviously on the biopharmaceutical use and this paragraph will be a brief exploration of the state of the art, concerning protein formulations.

Biocompatibility is the first concern when a substance is included in drug formulations. Native CDs induce membrane invagination and, at higher concentration, lysis in human erythrocytes. The haemolytic activity has been reported in this order β-CD>α-CD>γ-CD, that has inversed trend respect with the water solubility. [15][49] β-CD has the lowest aqueous solubility because it has been possible to detect a secondary belt formed by intramolecular hydrogen bonding between the secondary hydroxyl groups of neighboured glucose units.[15] To reduce the toxicological potential of CDs, in particular of β-CD which is reported to be nephrotoxic in some cases, the native structures have been derivatized. The aim of the derivatization is to obtain amorphous substances, highly water soluble and, of course, non-toxic molecules. The most widely used among derivatives are Randomly Methylated-β-Cyclodextrin (RM-β-CD) Sulfobutylether-β-Cyclodextrin (SBE-β-CD) and Hydroxypropyl-β-Cyclodextrin (HP-β-CD). These compounds have been shown great aqueous solubility and good binding capacity for poorly soluble drugs. [50] Table 1 reports the characteristics of some CDs, including those used in this study.

Table 1. Solubility (mg/mL) and molecular weight (Da) of main cyclodextrins[51]

Name of cyclodextrin	Solubility (mg/mL)	Mol. Wt. (Da)
Natural cyclodextrins		
Alpha cyclodextrin	145	972
Beta cyclodextrin	18.5	1135
Gamma cyclodextrin	232	1297
Chemically modified cyclodextrins		
Hydroxypropyl-β-cyclodextrin	≥600	1400
Sulfobutyl ether-β-cyclodextrin	≥500	2163
Randomly methylated-β-cyclodextrin	≥500	1312
Hydroxypropyl-γ-cyclodextrin	≥500	1576
Polymerized cyclodextrins		
Epichlorohydrin-β-cyclodextrin	>500	112000
Carboxy methyl epichlorohydrin beta cyclodextrin	>250	2000000–15000000

HP-β-CD is the most widely used among CDs and it is already on the market as drug carrier in the formulation of Sporanox by Janssen. [50] Its use has also been approved by Food and Drug Administration (FDA) in treatment of Nieman-Pick Type C (NPC) disease, a congenital neurodegenerative disorder.[52] This dissertation focus on the use as an excipient.

Cyclodextrins have proven to be effective against one of the most challenging issue in protein pharmaceuticals: aggregation. They act as artificial “chaperone”, preventing protein aggregation and promoting refolding. [31]

The mechanism by which cyclodextrins inhibit protein aggregation in solution could be the binding with exposed hydrophobic residues, limiting protein-protein interactions. Another possible mechanism, since CDs are essentially sugars, may be preferential exclusion. [15]

In the dried state CDs should protect the protein against unfolding through water replacement mechanism, in particular HP-β-CD. However, it is still unclear which are the stabilization mechanisms occurring during lyophilization. [15]

CDs, in particular the hydroxy-propylated form of β-CD, also showed good protection of proteins at the air-water interface during either agitation or freeze-drying. [15]

β-CD is the most effective among native CDs in inhibiting Amyloid-βpeptide aggregation, thanks to the inclusion of aromatic resides (phenylalanyl, tryptophan, tyrosine) in the cavity; this is a crucial result in studies concerning Alzheimer’s disease. [52]

Evidences of the inhibition of aggregation by inclusion of many exposed aromatic residues have been found in the study of interactions between β-CD and Recombinant Human Growth Hormone (Rh-GH). [15]

HP-β-CD protect ovalbumin and lysozyme against aggregation following denaturation at methylene chloride/water interface.[31] HP-β-CD has proven to have great stabilizing properties especially during freeze-drying: it is the most effective stabilizer examined for monoclonal antibody MN12 and it is also effective with mouse monoclonal antibody during storage at 56°C; it has been used to improve solubility and limit the formation of insoluble aggregates as consequence of lyophilization of interleukin-2 (IL-2) and the dimerization of lyophilized TNF during storage at 37°C. [12] The effectiveness of HP-β-CD as lyoprotectant has been widely studied for lactate-dehydrogenase (LDH) and it has been demonstrated to be more able to preserve the enzymatic activity than Polysorbate 80.

As previously mentioned, CDs may have also a detrimental effect on protein aggregation, enhancing the rate of the process. This phenomenon has been widely reported for SBE- β-CD, but in few studies also for HP-β-CD with oligomeric proteins glycogen phosphorylase b and glyceraldehyde-3-phosphate dehydrogenase (GADPH). [15]

4. Proteins used in this study

4.1. Granulocyte colony-stimulating factor (GCSF)

Granulocyte colony-stimulating factor (GCSF), marketed as Neupogen or Filgrastim, is a glycoprotein that has many functions in physiological and pathophysiological conditions. GCSF stimulates the production of granulocyte and stem cells and the release in bloodstream.
It regulates the production of neutrophilic granulocytes and it modulates neutrophil functions (survival, differentiation, proliferation) and the distribution of mature neutrophils and their precursors within the body.[53][54]

GCSF has proven to be effective in treating Chemotherapy Induced Neutropenia, a complication of myelosuppressive therapy, it is also used to treat congenital neutropenia, cyclic neutropenia, or idiopathic neutropenia. The protein, usually administrated through injection, reduces neutropenia manifestation like fever, infections, oropharyngeal ulcers.[17]

The effectiveness of GCSF has also been found in treating Parkinson's disease at early stages [18] and myocardium injuries, through the stimulation of migration of stem-cells from bone marrow and induction of their differentiation into cardiomyocytes or endothelial cells. [19]

The structure of GCSF has been determined by X-ray crystallography and 104 among the 175 residues are disposed in four-helical bundle structure.[55] The overall

dimensions of the protein are 45 Å x 26 Å x 26 Å [55] and its molecular weight is about 18110 Da. [17]

GCSF maintains structural and thermal stability at pH 2 and it is quite stable at pH 4.0. It is more sensitive to thermal denaturation when the pH increases.[56] The protein presents natural propensity to aggregation at pH 7.0 and 37 °C. [85]

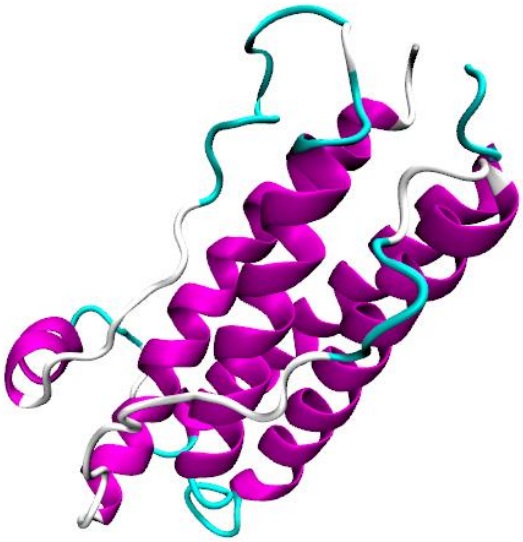


Figure 3 - Granulocytes Colony Stimulating Factor (GCSF) [23]

4.2. Lactate Dehydrogenase (LDH)

Lactate Dehydrogenase (LDH) is a globular tetrameric enzyme found in almost every tissue, especially in skeletal muscle, heart, liver, kidneys, brain, lungs, and red blood cells. It catalyses the conversion of lactate in pyruvate, reducing nicotinamide adenine dinucleotide (NAD) to (NADH) and release H+ ions. [57][58]

It has diagnostic use as indicator of cell damage. Determination of blood LDH level is used in the diagnosis of myocardial infarction, detection of skeletal muscle injuries, or in retrospective detection of lung problems. LDH is also used to monitor disease activity in cases of haemolytic and megaloblastic anaemias, thrombotic thrombocytopenic purpura, lung disease, tumours like particularly lymphomas and germinal cell cancers.[20][21]

A novel application of LDH lies in the production of biodegradable solvents through microbial conversion for biosynthesis of lactate esters from fermentable sugars. [22]

The structure of LDH includes 40% α-helices and 23% β-sheets and the hydrodynamic radius is about 4.2 nm. [59][60] The molecular weight of the protein is about 140 kDa.[61]

LDH has been chosen for this study because it is possible to find a lot of information in literature, about formulations and because of its reduced propensity to aggregation compared to GCSF.

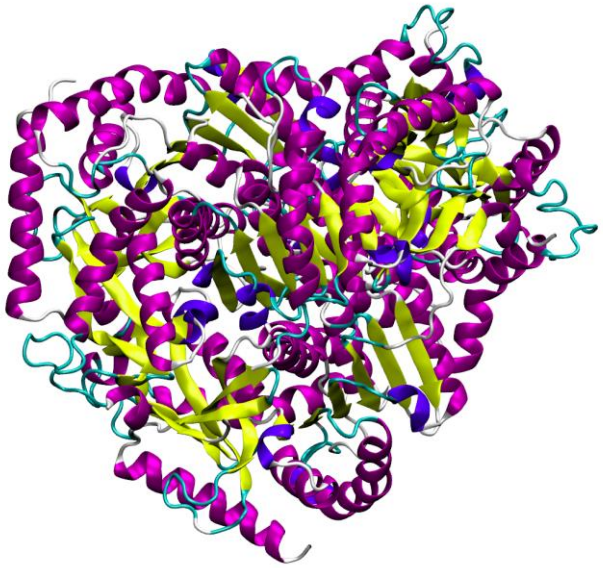


Figure 4 - Lactate Dehydrogenase (LDH) [23]

5. Motivation of this thesis project

The aim of this thesis project is the investigation of the use of cyclodextrins in protein formulation. The behaviour of GCSF and LDH, in formulations containing β-CD, HP-β-CD and maltoheptaose, which corresponds to the open form of β-CD is studied with several stability indicating methods. The comparison between closed and open form allows to distinguish the traits attributable to the CDs shape, which confers them a typical complexation ability.
The experimental plan has been elaborated in order to test the stability of LDH and GCSF proteins in presence and in absence of the aforementioned excipients, and the efficiency of CDs in the preservation of their native structure and activity when the protein is subjected to different stresses, which simulate, in a more pronounced way, the conditions that may occur during drugs preparation, shipping, storage.
The results shown in this manuscript result from a collaborative work between the Molecular & Pharmaceutical Engineering Laboratory lead by Prof. Dr. Roberto Pisano at Politecnico di Torino and the group of Prof. Dr. Gerhard Winter at Ludwig Maximilian Universität (LMU) of Munich. At Politecnico di Torino, it has been performed a series of Molecular Dynamics (MD) simulations, while the experiments, necessary to investigate and validate the results obtained and described in the following chapters, have been carried out by the Author at the Faculty of Pharmacy and Biopharmacy of LMU.

Chapter 2

Materials and methods

1. Materials

1.1 Proteins

1.1.1. Granulocyte colony-stimulating factor (GCSF)

GCSF (4 mg/mL) was provided in 10 mM sodium acetate buffer (ABS) at pH 4.0 with 5 % sorbitol and 0.004 % Tween 80. It was dialyzed against 10 mM sodium acetate buffer at pH 4.5. Dialysis was performed at 4°C in Spectra/Por® Dialysis Membrane (Standard RC Tubing, MWCO: 6-8 kDa; ThermoFischer Scientific, Waltham, Massachussets, USA) and the buffer was exchanged two times every 3 h, whereas the third dialysis step was carried out overnight. The concentration of GCSF after dialysis was determined by UV spectrometry at 280 nm with a NanoDrop 2000 Spectrophotometer (ThermoFischer Scientific, Waltham, Massachussets, USA) using an extinction coefficient of 0.86 mL/mg*cm.

1.1.2. Lactate Dehydrogenase (LDH)

LDH from rabbit muscle was purchased from SigmaAldrich (Lot nr. 36086922 and 36756721, Sigma-Aldrich, Steinheim, Germany). It was dialyzed against 10 mM potassium phosphate buffer at pH 7.0. Dialysis was performed at 4°C in Spectra/Por® Dialysis Membrane (Standard RC Tubing, MWCO: 6-8 kDa; ThermoFischer Scientific, Waltham, Massachussets, USA) and the buffer was exchanged two times every 3 h, whereas the third dialysis step was carried out overnight. The concentration of LDH after dialysis was determined using NanoDrop 2000 Spectrophotometer (ThermoFischer Scientific, Waltham, Massachussets, USA) with an extinction coefficient of 1.49 mL/ (mg cm). After dialysis the protein was diluted to a concentration of 1 mg/mL in the first set of experiments and to a concentration of 0.01 mg/mL for the second study of the activity of the protein.

1.2. Excipients and reagents

Table 2. Excipients and reagents list

Chemical	**Molar Weight (g/mol)**	**Description**	**LOT nr**	**Source**
Acetic Acid	60.05	p.a.	STBG8702	SigmaAldrich (Sigma-Aldrich, Steinheim, Germany)
β-Cyclodextrin	1134.98	Cavasol® w7		
Hydroxypropyl-β-Cyclodextrin	1541.5	Cavasol® w7 Pharmaceutical grade	60012210	Wacker Chemie AG (Burghausen, Germany)
Maltoheptaose	1153	Anomeric mixture	CYL-4301	CycloLab Ltd. (Budapest, Hungary)
Potassium dihydrogen phosphate	136.086	p.a.	A0104673 945	Merck KGaA (Darmstadt, Germany)
Potassium phosphate dibasic	174.18	p.a.	BCBD6301V	SigmaAldrich (Sigma-Aldrich, Steinheim, Germany)
Sodium Acetate	82.03	AnalaR® Normapur TM	13C290010	VWR International GmbH (Darmstadt, Germany)
Sodium Chloride	58.44	p.a.	18030010	Bernd Kraft GmbH (Duisburg, Germany)
Sucrose	342.3	p.a.	BCBV8251	SigmaAldrich (Sigma-Aldrich, Steinheim, Germany)

2. Methods

2.1 Preparation of the formulations

All protein formulations were prepared by spiking stock solutions of excipient in the respective buffer to the dialyzed protein solution to reach a final protein concentration of 1 mg/mL for GCSF in 15 mM excipient solution (if not otherwise stated) and 0.5 mg/mL for LDH in 10 mM excipient solution. In the second study of the LDH activity the protein concentration was adjusted to 0.005 mg/mL. All formulations were filtered through 0.2 μm Cellulose Acetate syringe filters (VWR International GmbH, Darmstadt, Germany). Highly purified water (Pure Water System; Sartorius Stedim Biotech GmbH; Goettingen) has been used in each step of the preparation. Both proteins were formulated with β-cyclodextrin, hydroxypropyl-β-cyclodextrin and maltoheptaose. LDH was also formulated with sucrose and sodium chloride to further investigate the activity of the protein.

2.2. Accelerated stability tests

2.2.1. Shaking

The shaking of the protein formulations has been carried out in the Thermomixer 5335 Comfort (Eppendorf, Hamburg, Germany). 1 mL of sample was filled in polypropylene centrifugal tubes (1.5 mL), placed vertically into the device and shaken at 1400 rpm for 1 hour and 5 hours, respectively, at 25 °C. The tubes were filled with 1 mL of sample in order to leave enough space for the formation of the air-water interface and be able to compare its effect in the presence or absence of the excipients.

2.2.2. Freeze-thawing

Freeze-thawing (FT) of the protein formulations has been performed in Epsilon 2-6D LSCplus freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany), on two set of samples. The first set has been subjected to a single FT cycle, the second one to 5 FT cycles. The cycles were programmed so that the samples could completely freeze/thaw: the freezing and the thawing step took both 1:10 h (freezing/thawing rate of 1°C/min) and they were separated by an intermediate step of 2:00 h in which the samples were kept at constant temperature. Freezing temperature has been set on -50 °C, while thawing temperature was set on 20 °C. The samples were prepared by filling 1 ml of each formulation in glass vials, placing them on the tray and filling the tray with other vials, in order to obtain a homogeneous temperature distribution.

2.2.3. Freeze-drying

Freeze-drying (lyophilization) of the protein formulations has been performed in an Epsilon 2-6D LSCplus freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany). The samples were prepared by filling 1 ml of each formulation in glass vials. The tray was filled with other glass vials, to obtain a great temperature

distribution. The temperature has been monitored with wireless sensors (by Martin Christ) placed in vials the middle and on the corner of the tray. The pressure has been monitored through a comparative pressure measurements system (Pirani pressor gauge and capacitive manometer). The following table reports the steps of the lyophilization cycle. At the end of the cycle, before opening the lyophilizer, atmospheric pressure was restored.

Table 3. Lyophilization steps

Step nr	Duration [hh:mm]	Final Temperature [°C]	Vacuum [mbar]
1	0:30	-3	-
2	1:00	-3	-
3	2:36	-50	-
4	2:00	-50	-
5	0:15	-50	0.09
6	1:00	-20	0.09
7	35:00	-20	0.09
8	4:10	5	0.09
9	1:40	25	0.09
10	7:00	25	2.00

2.2.4. Storage

The stability of the formulation has been investigated after storage at 4°C and 40°C. In particular, the analysis has been performed on the following samples:

- Stored at 4°C for 14 days
- Stored at 40°C for 14 days
- Stored at 4°C for 28 days
- Stored at 40°C for 28 days
- Stored after lyophilization at 40°C for 28 days.

2.3. Analytical Methods

2.3.1. Binding of the cyclodextrins and maltoheptaose to the protein

Biolayer Interferometry (BLI)

Biolayer interferometry (BLI) is a label-free technique based on the phenomenon of the interference of white light. BLI is used to measure the interaction of molecules. In the experiment, one molecule is immobilized on a biosensor. It is a tip dipped into a solution, containing the second molecule and the binding is detected. The solutions of interest are contained in a black 96 well plate. A change in the number of molecules bound, causes a change in interference, thus the signal can be correlated to the thickness of the coating. The signal is recorded in real time and reported as sensor gram.[62],[63]
Biolayer Interferometry (BLI) assay has been performed to detect the direct binding of the excipient to the protein in this study.

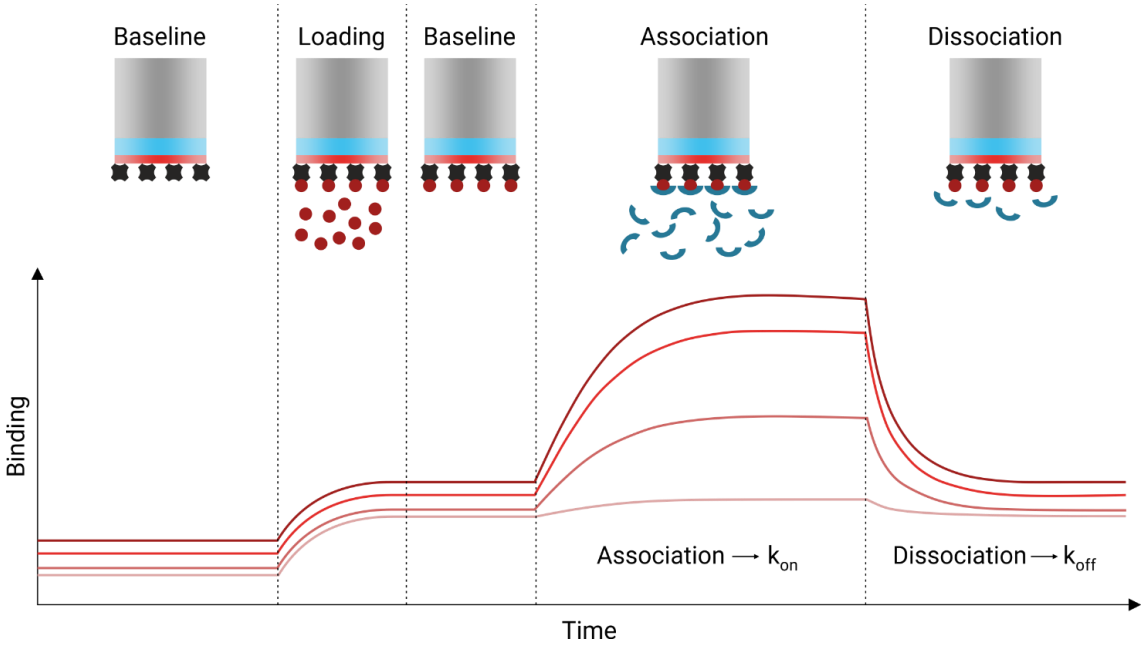


Figure 5 - BLI assay steps[64]

At the beginning of the assay, an initial baseline of the buffer is recorded. Subsequently, the biotinylated protein is immobilized on the sensor, which is then washed with the respective assay buffer to remove non immobilized protein and generate a new, stable baseline. Then the association with the excipient is recorded. The last step is the dissociation in presence of buffer.[65]
In these sets of experiments GCSF and LDH have been biotinylated and the immobilized on Super Streptavidin (SSA) biosensors. The experiments performed are:

1- Loading test of GCSF (0.06, 0.02, 0.0067 g/L) with sensors soaked in acetate buffer pH 4.5/ PBS buffer pH 7.5, using HP-β-CD as ligand (20 mM);
2- The same protocol as Exp. 1 but quenching with Biocytin after loading of GCSF and performing the measurements only at pH 4.5;

3- Binding test of GCSF (0.03 g/L) and reference sensors loaded only with biocytin VS HP-β-CD (100, 50, 25, 12.5, 0 mM) at pH 4.5;
4- Binding of GCSF (0.03 g/L) + reference sensors loaded only with biocytin VS HP-β-CD (20, 10, 5, 0 mM), β-CD (20, 10, 5, 0 mM), Maltoheptaose (50, 25, 12.5, 0 mM) at pH 4.5;
5- Loading test of LDH (0.06, 0.02, 0.0067 g/L) potassium phosphate buffer pH 7 using HP-β-CD as ligand (20 mM)
6- High loading test of LDH (0.12 g/L) for longer time.

The measurements were performed in the 8-channel Octet RED96e (ForteBio, Molecular Devices (Germany) GmbH; Biberach an der Riss, Germany). The results are expressed in terms of thickness (nm) of the molecular layer on the sensors over time. The time 0 s is fixed in correspondence of the association step.

Microscale thermophoresis (MST)

MST is a technique to quantify biomolecular interactions, based on thermophoresis, which is the movement of the molecule when subjected to a temperature gradient. The thermophoresis depends on size, conformation and hydration shell, allowing the user to quantify any change in those characteristics. During MST measurements, a temperature gradient is created through an infrared laser and the movement is detected and quantified via the intrinsic fluorescence. The solution to test is placed in capillaries (volume ~4μL). At the beginning, the molecules are homogeneously distributed, when the IR laser is turned on, a T-Jump (rapid change in fluorescence) is detected. When the laser is turned off, a reverse T-Jump is observed, which is linked to the back-diffusion.

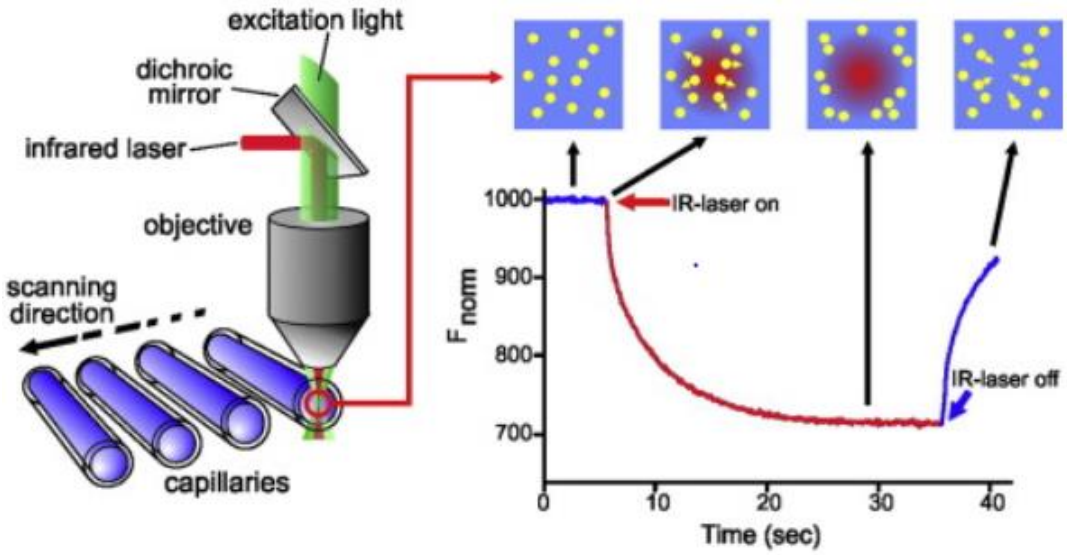


Figure 6 - Schematic representation of MST experiment

This technique has been used to investigate the protein-excipients binding, because the thermophoresis of a protein alone differs from thermophoresis of protein-ligand complex.

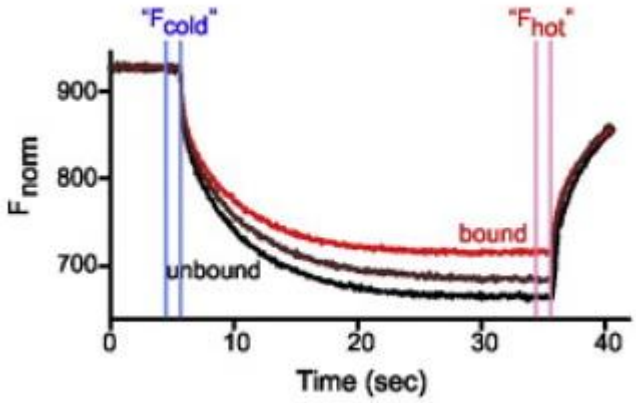


Figure 7 - General Behaviour of molecules fluorescence during MST experiment

The measurements have been performed in Monolith NT.Labelfree 201509-LF-N002 (NanoTemper Technologies, Munich Germany). The first experiment has been performed to compare the response of ABS, to ABS+excipient (HP-β-CD 10 mM), GCSF in ABS and GCSF in ABS+excipient. Proven the difference between the different samples, the behaviour of GCSF when formulated with different concentrations of β-CD, HP-β-CD and maltoheptaose has been investigated. The concentrations tested are: 10, 5, 2.5, 1.25, 0,625, 0.3125, 0.15625, 0.078125 mM. Except for the first experiment, in the Results and Discussion section are reported the results of experiments performed with premium capillaries, because the response obtained with normal capillaries was neither clear nor satisfying. Each measurement has been performed in duplicate.

2.3.2. Protein aggregation and conformational stability

PH measurements

The pH, as previously discussed, affects the protein stability. Thus, it has been measured in each sample, in the initial condition, after stress and after storage.
The pH has been measured using the MP225 General Purpose GLP pH /mV / T Meter (Mettler Toledo, Columbus, Ohio, USA) at room temperature.

Circular Dichroism

Circular dichroism is the measurement of the differential absorption of left and right circularly polarized light. To discriminate between the two forms of light, a molecule must be chiral. CD spectroscopy can be performed on proteins, given the fact that proteins possess chromophores able to give rise to a CD signal. The technique involves

absorption in the far (180–250 nm) and near-UV (250 nm– 180 nm) and provides protein secondary and tertiary structure information. The far-UV region corresponds to peptide bond absorption and the spectrum can be analysed to investigate the content of regular secondary structure features (e.g.: α-helix and β-sheet). Near-UV provides direct access to aromatic amino acid side residues, giving information about the tertiary structure of the protein.[66][67]
Circular Dichroism measurements have been performed to understand whether the secondary and the tertiary structure of the proteins are affected by the excipients.
The measurements were performed in the Jasco J-810 Spectropolarimeter (Jasco Corporation, Tokyo, Japan), using the Spectra Manager program. The device has been purged with N_2 for about 15 minutes before the measurements started. The liquid sample of 1 mL is placed in a high-performance quartz glass cuvette, with 10 mm light path for the near-UV and 0.1 mm light path for far-UV (High Precision Cell; Hellma Analytics). The cuvette must be properly cleaned with 2 % Hellmanex solution before putting the sample inside. Each formulation has been measured right after preparation without applying any stress on the sample, in order to detect the eventual change in structure caused by the presence of cyclodextrins or maltoheptaose in the formulations. The absorbance and thus the ellipticity has been measured in the near-UV region for GCSF and near and far-UV region for LDH. CD data are presented in terms of either ellipticity (degrees) or absorbance. Molar ellipticity (θ) is corrected for concentration. The units of molar ellipticity are historical (deg×cm2/dmol). The mean residue weight (MRW) is calculated from the molecular mass/ (N – 1); N = number of amino acids, treating the proteins as a solution of amino acids. For most proteins, MRW is around 110.

$$\theta_{mrw} = \frac{MRW \cdot \theta_{obs}}{1000 \cdot c \cdot l}$$

Where:

- θobs = observed ellipticity [mdeg]
- MRW= mean residue weight
- l = pathlength [cm]
- c = concentration [mg/ml]

Differential Scanning Fluorimetry (nanoDSF)

NanoDSF is a label-free technology, which used to investigate the thermodynamic stability of proteins. It applies a thermal gradient on the protein solution to detect the change in intrinsic fluorescence the aromatic sidechains of tyrosine and tryptophan residues. The temperature increase causes the unfolding of the protein that leads to an exposure of those residues, which are usually buried into the core of the protein, to the solvent. Thus, microenvironment polarity around tryptophan residues changes and the fluorescence detected with the dual-UV detection system (330 nm and 350 nm) is different.[68]
Detecting the back-scattering signal, this method allows the user to measure the aggregation temperature (onset temperature at which aggregates first appear) and the apparent melting temperature (temperature at which the protein is about 50%

denatured); it is called “apparent” because it could differ from the T_m measured with DSC.[68][69]
The aim of the measurements is to detect the difference in thermal stability due to the presence of excipients
The measurements were carried in the Prometheus NT.48 (NanoTemper Technologies, Munich, Germany) with PR. Thermal Control Software, in 10 µL high-quality capillaries. The intensity of the radiation applied has been selected after a preliminary scanning and has been set on 30% for GCSF and 20% for LDH. The device applies a temperature ramp from 20°C to 90°C, with a gradient of 1°C/min. The ratio of the integrated fluorescence 350/330 nm and the scattering have been measured for each capillary at a rate of a few tenths of a second, while the temperature was increasing. The T_{agg} is given by the onset of the ratio, while the T_m is given by the onset of scattering.
The samples have been measured in triplicate, right after the preparation of the different formulations, avoiding the inclusion of air in the capillary. The data have been evaluated as mean and standard deviation of the three capillaries for each formulation.

Isothermal Chemical Denaturation (ICD)

GCSF stock solutions of 1 mg/mL, 10 M urea stock solutions as well as the buffers were prepared either with or without excipient. The Viaflo Assist and 12.5 µL/ 125µL Viaflo pipettes (Integra Biosciences, Konstanz, Germany) were used to add 8 µL of each GCSF stock solution, the respective amount of buffer and subsequently the 10 M urea stock solutions in the respective buffer in a non-binding 384-well plate (Corning, USA) (Table X). Mixing was performed manually with new tips and the plate was sealed with an EASYseal sealing film (Steinheim, Germany) and incubated for 1 and 17 hours at room temperature. All samples were measured in triplicates. A FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany) was used to measure the intrinsic protein fluorescence at 330 and 350 nm after excitation at 280 nm. The ratio of fluorescence intensity 350nm/330nm was calculated for each urea concentration and fitted to a two-state model with the CDpal software[70]. The fit provided C_m- and ΔG-values.

Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) is a useful method to study the diffusion behaviour of macromolecules in solution. When the light beam encounters the molecule, it scatters as function of dimension, size and shape of the molecule. In DLS the time fluctuation in the intensity of the scattered light, due to Brownian motion of molecules, is useful to obtain the autocorrelation function, which is related to the translational diffusion coefficient. The spectrum and the autocorrelation function provide information about the particle size and distribution.[71],[72],[73]
The measurements were carried out in the DynaPro Platereader III (Wyatt Technologies, Santa Barbara, California, USA), which enables the user to detect aggregation, aggregate size distributions, changes in solution molecular weight, and

propensity for aggregation.[74] Furthermore, for this work the indicator of colloidal stability k_D has been measured.

Before performing the measurement, the following steps have been done:

1. Centrifugation of 200 µL of each sample for 10 minutes at 10000 rpm in Sigma 4K15 Table Top Centrifuge (Sigma Laboratory Centrifuges, Osterode am Harz, Germany);
2. Allocation of three 30 µL aliquots of each sample in a black 384 well plate (Corning™ Low-Volume 384-Well Black Flat Bottom Polystyrene Non-Treated Microplate; ThermoFischer Scientific, Waltham, Massachussets, USA);
3. Centrifugation of the plate for 2 minutes at 2000 rpm in Heraeus Megafuge 16R centrifuge equipped with an M-20 well plate rotor (Thermo Fisher Scientific, Wilmington, NC);
4. Sealing of each full well with silicon oil;
5. Centrifugation of the plate for 2 minutes at 2000 rpm in Heraeus Megafuge 16R centrifuge equipped with an M-20 well plate rotor (Thermo Fisher Scientific, Wilmington, NC).

The measurements have been performed in triplicate for each sample, in order to obtain reliable data. The results may be deeply affected by the presence of air bubbles in the well, thus any pipetting mistake should be avoided and the device has been set in a way to take a picture of each well, in order to verify the presence of bubbles or dust on the bottom of the plate. The temperature has been kept constant at 25 °C. Each measurement was performed in triplicates and with 10 acquisitions of 5 s. The radius and the polydispersity were evaluated as mean of triplicate, excluding measurements where the data showed no consistency with the autocorrelation function. For each well the device provides average values of radius and PD%, calculated on the different acquisitions.

The interaction parameter k_D was determined from the slope of the diffusion coefficient depending on the protein concentration (1 – 4.5 mg/mL). Each measurement was performed in triplicates and with 10 acquisitions of 5 s.

Fluid Imaging Microscopy (FlowCam)

The Flow Cytometer and Microscope (FlowCam) can perform a rapid detection and identification of particles in liquid samples. Flow imaging microscopy uses camera with high magnification to capture and save images of the sample solution passing through a flow cell. The sample is pumped into the cell by a syringe pump. Different flow cells are used for different magnifications. [75],[76],[77]
The measurements were carried out in a FlowCam 8100 (Fluid Imaging Technologies Inc., Scarborough, Maine, USA) in AutoImage Mode, with 10X magnification. This mode captures images of the flowing fluid at regular intervals. It is used for samples with high particle concentrations.[77]
The VisualSpreadsheet Software provides real-time images and enables their analysis. The parameters, like size, are measured from each image and then the statistical analysis is performed with the software. The images enable the user to distinguish between aggregates, contaminants and air bubbles. [75],[76],[77],[78]
For each run, 150 µL sample were injected into the FlowCam, with a flow rate of 150 µL/min, detection time of 60 seconds. Imaging and flow settings were properly adjusted, in order to capture the particles in the optimal way: the distance to the nearest neighbour has been set to 3 µm, the thresholds were set in a way that the device does not take the background into particles account and counts all the particles in the sample, as well (13.00 dark pixels, 10.00 light pixels). The maximum efficiency has been set on 72%, in order to not count each particle more than once, since the motion in the cell is in laminar regime. The concentration of particles in the samples is automatically calculated dividing the number of particles counted by the total volume of the sample. The particles counted are divided in four size ranges, to easily distinguish the eventual presence of aggregates. The measurements have been performed in triplicate. The issues in the use of the device were the inclusion of air bubbles and the huge standard deviation observed for many samples.
The number of particles has been also measured for the buffers and for buffer containing β-cyclodextrin at different concentrations.

Turbidity

The turbidity of the acetate buffer and of potassium phosphate buffer containing β-cyclodextrin at different concentrations has been measured in order to find the optimal concentration. The aim of the measurements, coupled with the data collected in the FlowCam, is to find a concentration where the β-cyclodextrin is completely soluble and high enough to allow an effect on the protein. The measurement has been carried out in the FLUOstar Omega multi-mode microplate reader (BMG Labtech, Ortenberg, Germany) at 350 nm, using the Hellma Quartz 96 well plate.

2.3 Monomer recovery for GCSF

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

The liquid chromatography is a technique used to separate and quantify the components or solutes in a mixture. RP-HPLC is performed in a glassy or metallic thin packed column, in which the solvent is forced through to progressively elute the sample. The column contains particles with molecules bonded to their surface, forming a liquid like film on it. In RP-HPLC the solvent constitutes the mobile phase and has polar characteristics, while the liquid coated particles are the stationary non-polar phase.[79]
Thus, the species sooner eluted are the ones that show the highest affinity to the mobile phase, with respect to the species later eluted.
A detector is placed at the exit of the column and is connected to a readout device (computer). The eluted component appears as peak on the chromatogram, which is the plot of the response over time. The process is carried out with the gradient elution method, in which the concentration of solute in the mobile phase is increased during the run, modifying the polarity.[79] In this kind of technique the volume of the sample is usually deeply smaller than the volume of the eluent.[80]
RP-HPLC has been used only for GCSF formulations to calculate the monomer recovery of the protein after stress in with respect to the initial formulation. The samples were centrifuged (10000 rpm for 10 minutes) before being measured. The machine provided by Dionex (ThermoFischer Scientific, Waltham, Massachussets, USA) has the following configuration:

- Solvent Rack SOR-100
- P680A HPLC Pump
- ASI-100 Automated Sample Injector
- Thermostated Column Compartment TCC-100
- RF-2000 Fluorescence Detector.

The column used is the BioBasic-18 (ThermoFischer Scientific, Waltham, Massachusetts, USA), with dimensions 250X2.1 mm, packed with 5µm diameter particles. The eluents were: 0.1 % w/v Trifluoroacetic acid (TFA) and 0.1 % w/v Acetonitrile (ACN) in highly purified water (Eluent A) and 0.1 % w/v TFA in ACN (Eluent B). The gradient applied was 40-80% of Eluent A to B. The amount of sample injected was 60 µL, with a flow rate of 0.2 ml/min GCSF eluted within 16-24 minutes, as revealed by absorbance measured at 214 and 280 nm.
The column oven temperature has been set on 37 °C. The protein was detected with UV detector. The software used to set the analysis and analyse the data is Chromeleon™ Chromatography Data System (CDS) Software, and the recovery for each formulation was calculated referring the average peak area of the stressed sample to the area of the liquid ones.

2.4 Activity Assay for LDH

Colorimetric assay

The enzymatic activity of LDH, in the reduction reaction of NAD to NADH, is determined by monitoring the increase of absorption at 450 nm. The assay has been performed using the kit provided by Sigma-Aldrich (Sigma-Aldrich, Steinheim, Germany). The formulations have been diluted to a concentration of 0.1 µg/mL of protein (last dilution with LDH assay buffer), because the activity must be within the linear range defined by the NADH standard curve. This concentration has been selected after preliminary tests: 0.25-0.75 µg/ml, 0.01-0.21 µg/ml, 0,04-0,06 µg/ml and the last test to compare the activity of a protein in stock since 2016 and a newly purchased one. Duplicates of each sample (50 µL each) were added in a clear 96 well plate and mixed with 50 µL of master reaction mix. The plate has been incubated at 37 °C for 2 minutes and gently shaken before taking the initial measurement. The measurements have been performed in the FLUOstar Omega multi-mode microplate reader (BMG Labtech, Ortenberg, Germany). The absorption was recorded for 10 minutes and afterwards the increase in absorption was calculated by the following equation:

$$\Delta A_{450} = \Delta A_{450,final} - \Delta A_{450,initial}$$

Comparing the ΔA_{450} to the standard, the amount of NADH generated has been calculated. Thus, the activity has been determined by the following equation:

$$LDH\ Activity = \frac{B}{Reaction\ Time \cdot V} \cdot f$$

Where:

- B= amount of NADH generated [nmol];
- Reaction time= t_{final} - $t_{initial}$ [minutes];
- V= volume of sample [mL];
- f=dilution factor

The assay has been performed in order to check whether the activity of the protein is affected by the stresses performed on the formulation and if the protein is protected by the excipients.

Chapter 3

Results and discussion

1. GCSF

1.1. Binding of the cyclodextrins and maltoheptaose to the protein

1.1.1. BLI

The first experiment has been performed in order to determine the proper concentration and test the loading of the protein. Loading with acetate buffer pH 4.5 and highest concentration of GCSF (0.06 g/L) gave the best loading signal. However, the HP-β-CD used as ligand showed an unspecific binding signal. The experiments have been repeated with an additional quenching step with Biocytin after loading the protein and the unspecific binding was still visible but weaker; thus, all further experiments have been performed including the quenching step.

The figures 8, 9, 10 show the thickness of the biolayer during the association step of β-CD, HP-β-CD and maltoheptaose to GCSF at pH 4.5. The intensity of the signal, thus the thickness of the biolayer, was low for β-CD but it a slight signal increase in a concentration dependent manner was observed. The fact that the signal was more intense for HP-β-CD and the sensor grams for the 5- and 20-mM solutions suggest a concentration dependence, however the 10 mM showed opposite behaviour and should not be considered. The maltoheptaose has given weirdly shaped binding curves and showed a drop within association phase and increase during dissociation, the highest concentration had the lowest signal and the intensity was higher than β-CD.
In molecular dynamic simulations the binding mechanism identified is preferential inclusion and the best interaction was expected for β-CDs [23], while the signal given by the Octet is the weakest one for β-CD solutions.
Furthermore, the maltoheptaose has been included as negative control, because it does not have the truncated hollow conical shape typical for cyclodextrins, which should favour the binding, and because the excipient, according to the conducted simulations, should be less included[23]. Thus, the intensity values were unexpected.

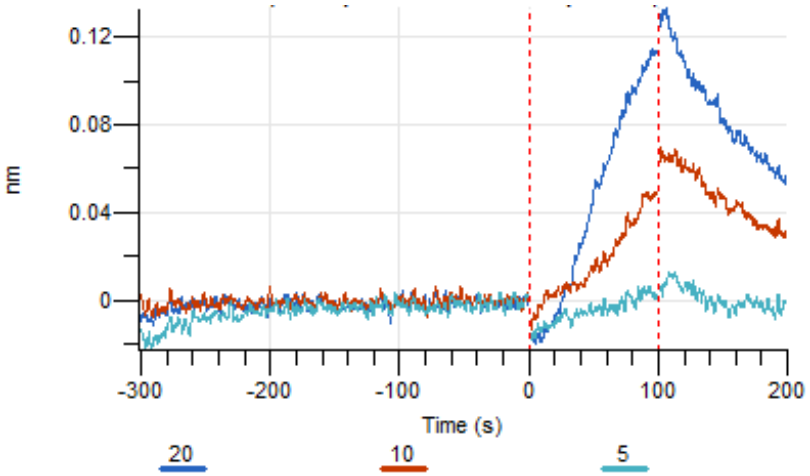


Figure 8 – Biolayer thickness as function of time for GCSF pH 4.5 in β-CD 20 mM (blue), 10 mM (red), 5 mM (light blue)

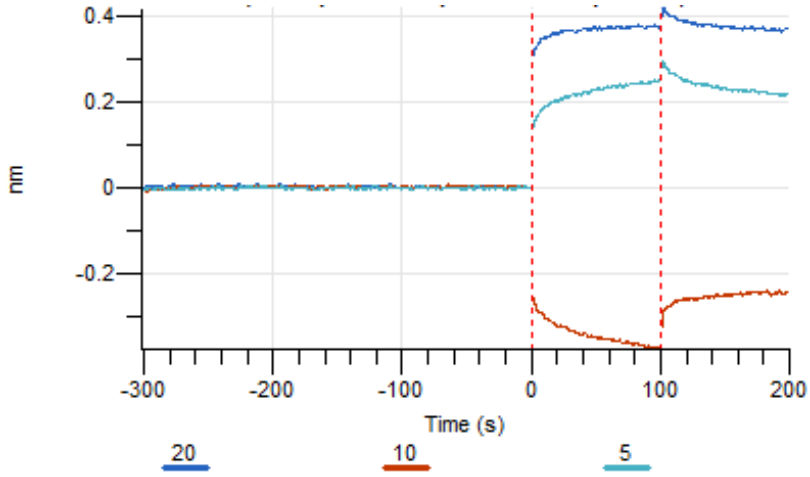


Figure 9 - Biolayer thickness as function of time for GCSF pH 4.5 in HP-β-CD 20 mM (blue), 10 mM (red), 5 mM (light blue)

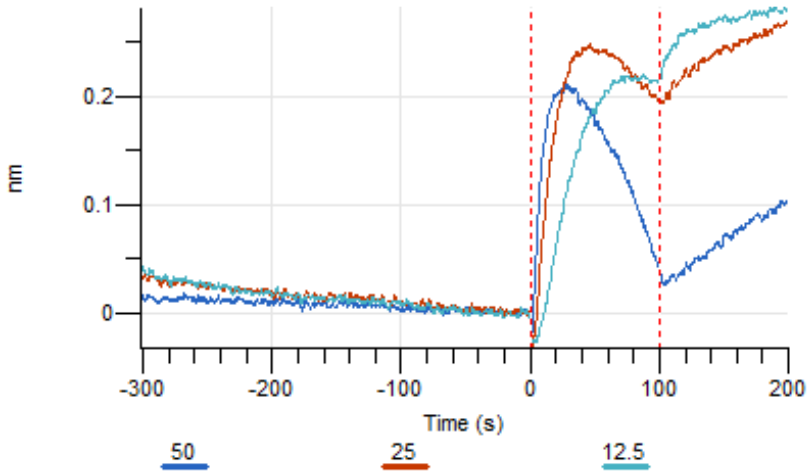


Figure 10 - Biolayer thickness as function of time for GCSF pH 4.5 in maltoheptaose 50 mM (blue), 25 mM (red), 12.5 mM (light blue)

The figure 11 depicts the loading test of GCSF at pH 7.5. There has not been any binding signal of the excipient to the protein and the values were even negative after baseline correction with increasing excipient concentration. Since no satisfying immobilization signal could be achieved for GCSF at pH 7.5, no further excipients were investigated in this condition.

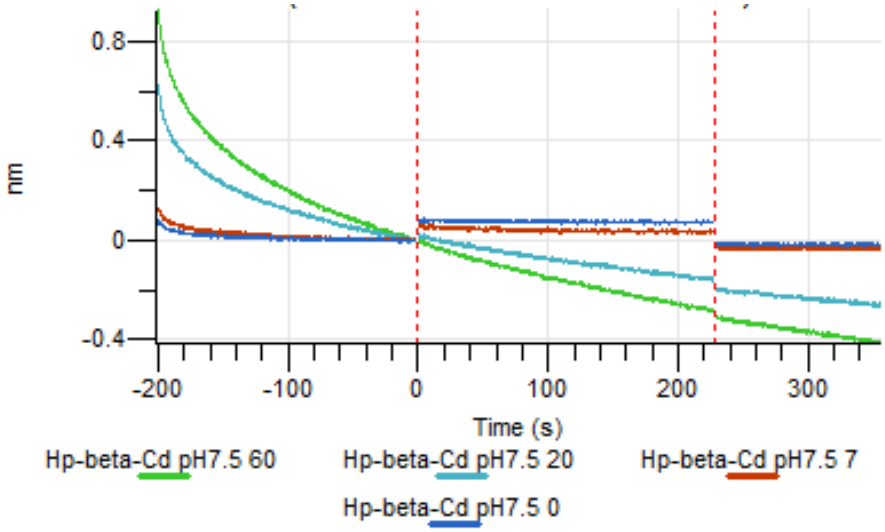
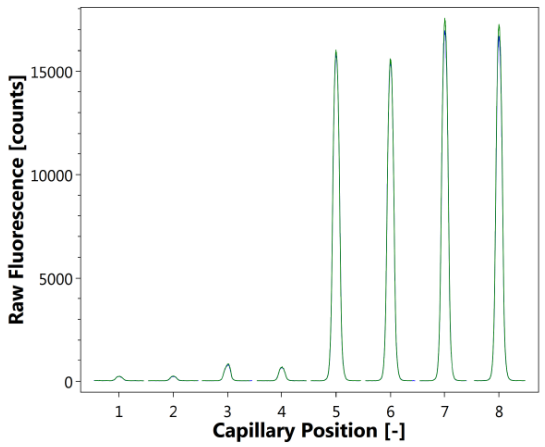


Figure 11 - Biolayer thickness as function of time during loading test of GCSF pH 7.5 in HP-β-CD 60 mM (green), 20 mM (light blue), 7 mM (red), in ABS (blue)

1.1.2. MST

The first experiment, performed in normal capillaries, has been used to estimate the different response given by ABS containing excipient or not, and protein with and without excipients.



Cap. nr	Target
1	ABS
2	ABS
3	ABS+HP-β-CD 10 mM
4	ABS+ HP-β-CD 10 mM
5	GCSF
6	GCSF
7	GCSF+ HP-β-CD 10 mM
8	GCSF+ HP-β-CD 10 mM

Figure 12 - Raw Fluorescence in each capillary

For this preliminary experiment the normalized fluorescence is not shown, being the signal noisy in absence of protein and thus in absence of fluorophores.

Figures 13, 14, 15 show the normalized fluorescence as function of time during the experiments involving HP-β-CD, β-CD and maltoheptaose. In each experiment the concentrations reported in the Materials and Method section have been tested in duplicate.

For HP-β-CD, relative fluorescence increased in concentration manner. This behaviour suggests that the protein binds more with the excipient when its concentration increases. This is consistent to what has been found in BLI experiments and to the expectations: the higher the concentration, the higher the probability of protein and excipient to meet each other and consequently bind.

For β-CD it was possible to see binding only in correspondence of the highest concentration (10 mM), while for maltoheptaose there was no difference for different concentrations and the signal is noisier. However, the relative fluorescence was higher in the case of the open form of β-CD, compared to the closed one, this is somehow in accordance with what has been observed in the BLI.

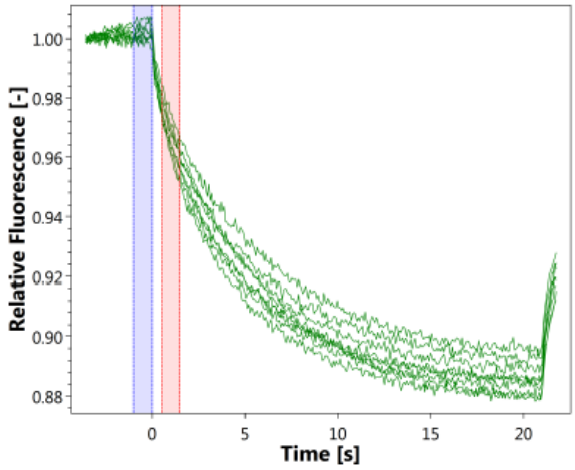


Figure 13 - Relative Fluorescence as function of time for GCSF formulated with HP-β-CD in ABS

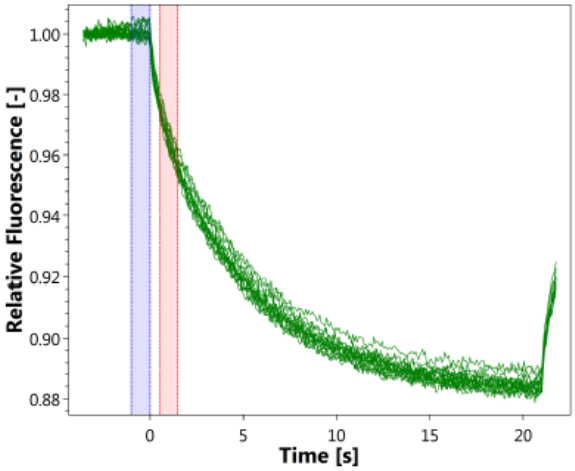


Figure 14 - Relative Fluorescence as function of time for GCSF formulated with β-CD in ABS

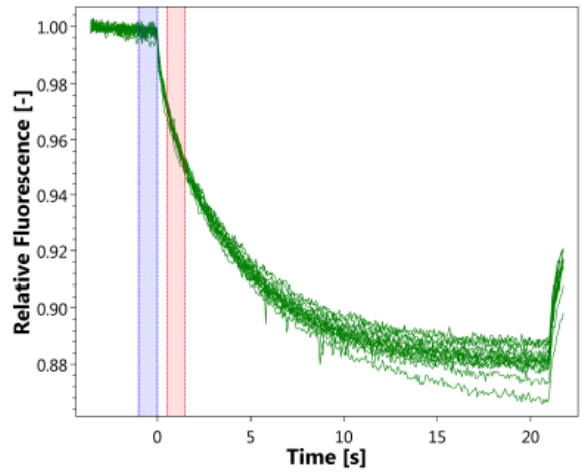


Figure 15 - Relative Fluorescence as function of time for GCSF formulated with maltoheptaose in ABS

1.2. Protein aggregation and conformational stability

1.2.1. pH

The pH detected in each sample right after formulation and after accelerated stability tests and storage. The value was 4.6±0.09 and it has not significatively changed except in lyophilized samples and stored lyophilized samples, were the pH shifted to 7 in formulation without any excipient, to 5.4 in formulation with β-CD (open and closed form) ant to 5 in formulation with HP-β-CD. This is due to the sublimation of acetic acid from buffer.

1.2.2. Circular Dichroism

The figure 16 shows the ellipticity of the formulations in the near-UV region. This analysis is useful to investigate the tertiary structure of the protein and whether it is affected by the addition of cyclodextrins and maltoheptaose.
Concerning the β-CD and HP-β-CD, there was not significant change in tertiary structure. The maltoheptaose formulation presented unclear behaviour: the spectrum is shifted but the shape is unchanged, this might be due to aggregation, although, being the shape unaltered, it seems that the environment of the aromatic residues stays unchanged.

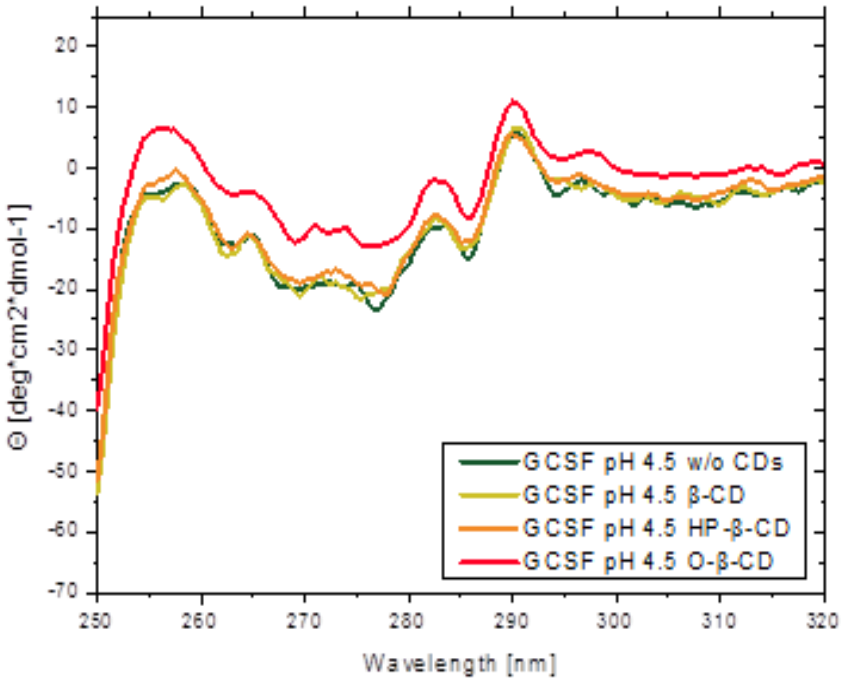


Figure 16 - CD spectrum in the near-UV region: molar ellipticity as function of wavelength for GCSF pH 4.5 in ABS without any excipient (green), with β-CD (yellow), with HP- β-CD (orange), with maltoheptaose (red)

Figure 17 shows the formulations spectra in far-UV region. The measurements were performed to investigate the secondary structure of the protein. The spectra of excipient containing formulations suggest an increase in alpha-helical content. The stabilizing function of cyclodextrins has been documented: Bovine Serum Albumin tends to refold in presence of the β-CD[82], HP-β-CD stabilizes the conformation of deslorelin.[83] The spectra presented two minima around 222 and 208 nm, typical values of proteins with an alpha-helical structure, and consistent with that expected for GCSF from literature.[84][85]

However the results are not convincing because the spectrum of GCSF in literature presents peaks with lower ellipticity values[86] and, in the analysed samples, the protein had not been subjected to any stress that could cause conformational change.

It was not expected that the cyclodextrins have such an impact on the structure of GCSF, especially since the NUV spectra only showed minor deviations between the different formulations. Furthermore, other studies evidenced that the addition of surfactants increases the ellipticity value [86], while here the behaviour is opposite and needs to be further investigated.

Furthermore, other studies evidenced that the addiction of surfactants increases of the ellipticity value [84], while here the behaviour is opposite and needs to be further investigated.

It may also be related to the protein concentration, on which the measurements depend, because slight deviations may change the intensity of the spectra.

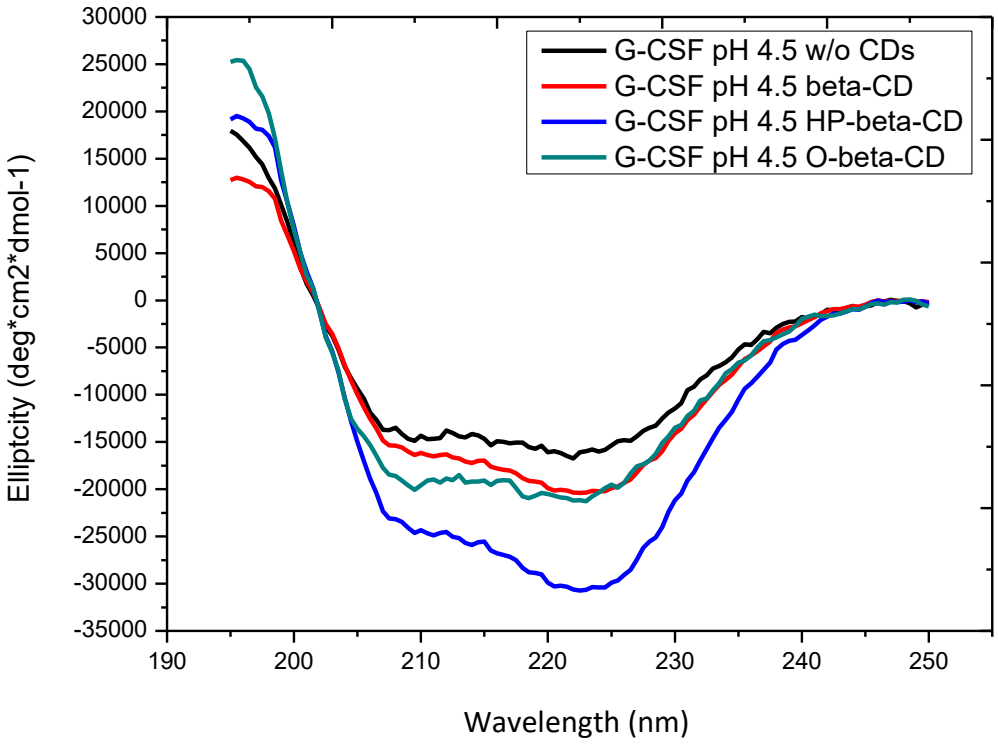


Figure 17 - CD spectrum in the far-UV region: molar ellipticity as function of wavelength for GCSF pH 4.5 in ABS without any excipient (black), with β-CD (red), with HP- β-CD (blue), with maltoheptaose (green)

1.2.3. NanoDSF

In the figure 18 the ratio of integrated fluorescence is shown and the data points of the four different formulations were almost coincident. The transition of the protein from the folded to the unfolded state occurs nearly at the same temperature for all formulations.
The figure 19 shows the intensity of the scattering, which was higher for the formulation of GCSF without any excipient in the temperature range 70-90°C.
The results are clarified in the table 3.1 where T_m and T_{agg} are reported. Both temperatures were a little higher when the HP-β-CD is added, although the difference with respect to the other formulations is about 1°C: aggregation temperature is around 55°C for each formulation, while is about 56°C when HP-β-CD is added, melting temperature is 56°C for each formulation and 57°C with the aforementioned excipient. Thus, it can be assumed that HP-β-CD confers thermal stability to the protein, offering slightly greater protection against unfolding due to temperature rise but it is necessary to further investigate the topic.

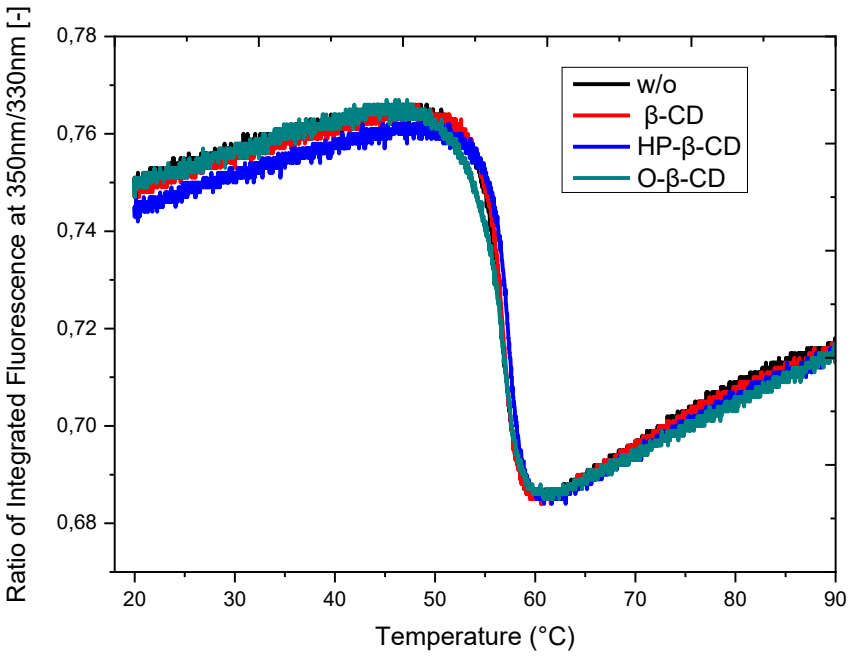


Figure 18 - Ratio of integrated fluorescence at 350nm/330nm as function of temperature for GCSF pH 4.5 in ABS without any excipient (black), with β-CD (red), with HP- β-CD (blue), with maltoheptaose (green)

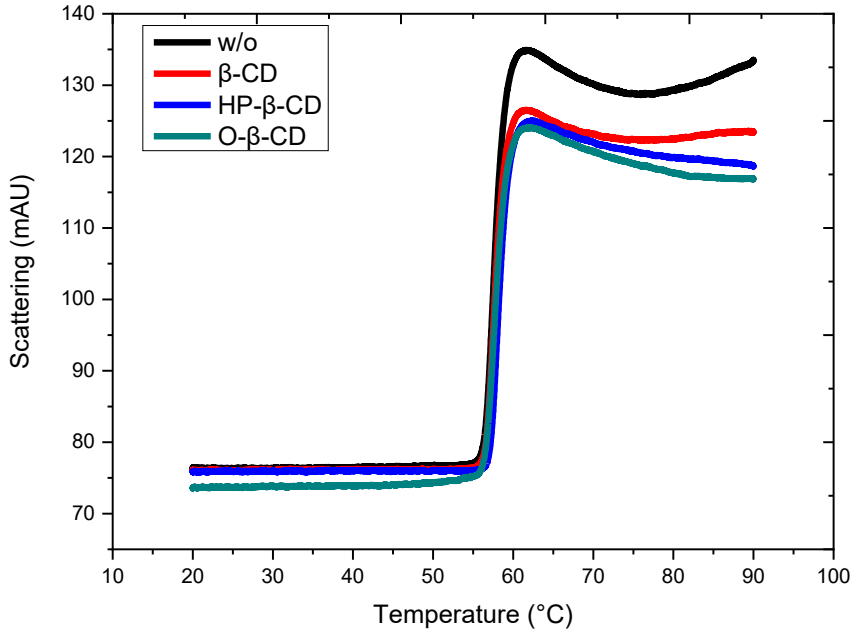


Figure 19 - Scattering intensity as function of temperature for GCSF pH 4.5 in ABS without any excipient (black), with β-CD (red), with HP- β-CD (blue), with maltoheptaose (green)

Table 3. Aggregation temperature and melting temperature of GCSF formulations

Formulation	**T_{agg} (°C)**	**Err**	**T_m (°C)**	**Err**
GCSF 15 mM ABS pH 4.5	55,01	0,08	56,49	0,02
GCSF 15 mM β-CD	55,32	0,30	56,60	0,02
GCSF 15 mM HP-β-CD	56,02	0,07	57,16	0,01
GCSF 10mM O-β-CD	54,14	1,39	56,45	0,16

1.2.4. ICD

The figure 20 shows the ratio of integrated fluorescence for different concentration of urea. Given that it is quite difficult to distinguish the behaviours of the formulation in this kind of plot, the change in Gibbs free energy of unfolding it is also reported. It does not indicate any significant difference between the formulations. While the concentration of denaturing agent necessary to unfold the protein is slightly higher when HP-β-CD is used as excipient. Thus, in formulations containing HP-β-CD the protein seems to be a little more stable than in the other investigated formulations.

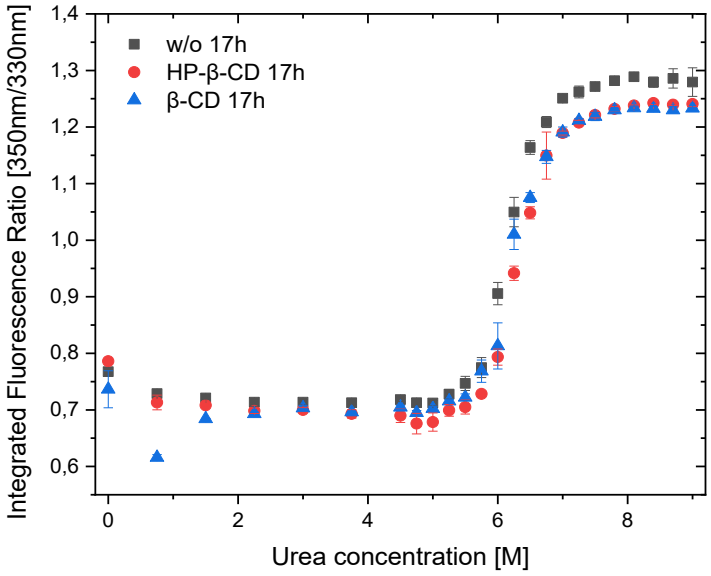


Figure 20 - Integrated fluorescence ratio as function of urea concentration measured after 17 h of incubation at room temperature for GCSF pH 4.5 in ABS without any excipient (black square), with β-CD (blue triangle), with HP- β-CD (red dot)

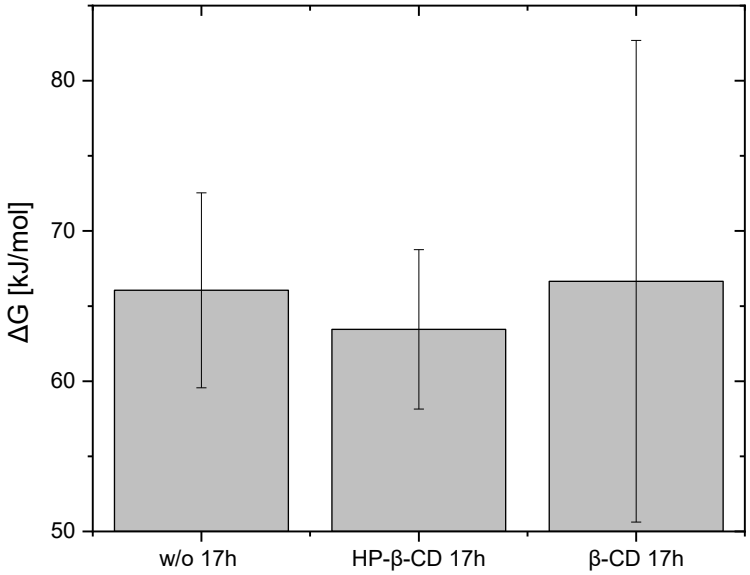


Figure 21- Change in Gibbs free energy of unfolding for GCSF pH 4.5

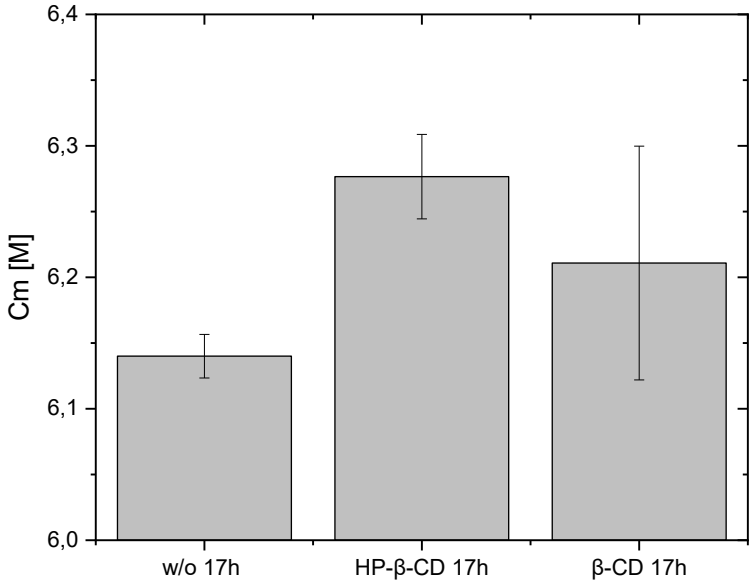


Figure 22 - Urea melting concentration for GCSF pH 4.5

1.2.5. DLS

The aggregation temperature has been measured by dynamic light scattering too. There was no appreciable difference between the formulation without any excipient and the one with HP-β-CD, while the aggregation temperature is lower when β-CD is added. However, the difference is less than 1°C. The values provided by this kind of measurements were lower than T_{agg} detected with nanoDSF, but close to what is possible to find in literature for similar concentration of GCSF in the same buffer at pH 4.[84] Please note that the formulation labelled as "water" is protein in acetate buffer, without any excipient.

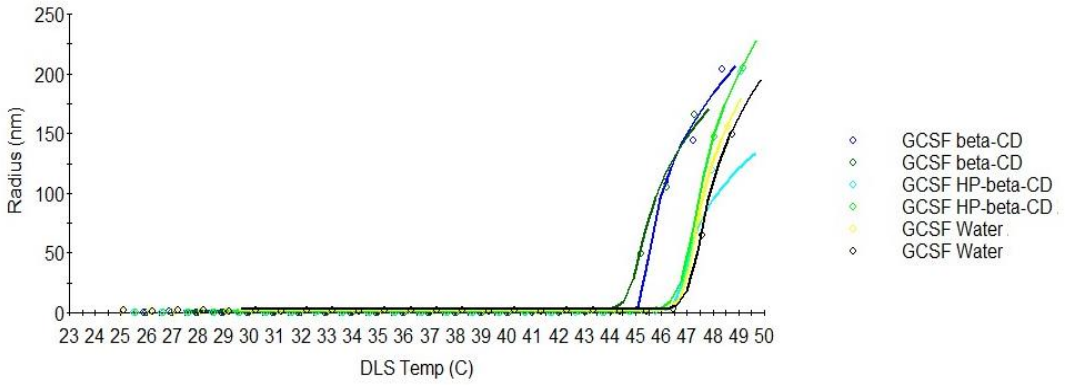


Figure 23 - Hydrodynamic radius of the protein as function of temperature for GCSF pH 4.5 in ABS without any excipient (black and yellow), with β-CD (blue and dark green), with HP-β-CD (light blue and green)

Table 4. Aggregation temperatures

Formulation	**T_{agg} [°C]**	**Error**
GCSF 10mM ABS pH 4.5	45,94	**0,07**
GCSF 10mM β-CD	44,30	**0,60**
GCSF 10mM HP-β-CD	45,97	**0,61**

The dynamic light scattering has been used to investigate the colloidal stability as well, obtaining the following k_D values:

Table 5. kD values

Formulation	**k_D (ml/mg) *10^{-2}**
GCSF 10mM ABS pH 4.5	-2.47
GCSF 10mM β-CD	-8.27
GCSF 10mM HP-β-CD	-7.38

Negative k_D values indicate the presence of attractive forces, thus a tendency to aggregation. It is also possible to note that the propensity to aggregate is enhanced by the presence of cyclodextrins.
The fluctuations in intensity of scattered light can be related to diffusion coefficient.[87] It has been measured in the three different formulations selected for this preliminary analysis, then the linear regression provided the function which correlate the diffusion coefficient itself and the concentration of the protein.

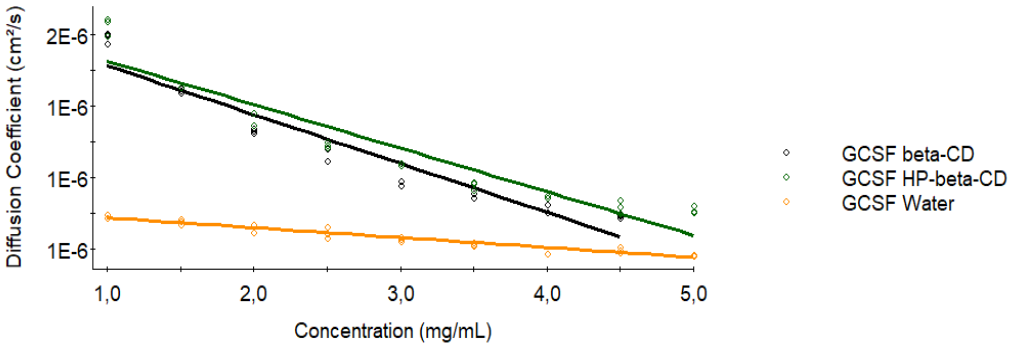


Figure 24 - Diffusion coefficient as function of concentration temperature for GCSF pH 4.5 in ABS without any excipient (yellow), with β-CD (black), with HP- β-CD (green)

The infinite dilution diffusion coefficient (D intercept for c=0 mg/mL) was different for each formulation. This means that there is a signal given by cyclodextrins, which diffuse faster than the protein. In fact, in literature it is possible to find the value of

infinite dilution diffusion coefficient of β-CD in aqueous solution: $3.210*10^{-6}$ cm^2/s [88], which is higher than the value found for GCSF in our experimental measurements, in absence of excipients. Increasing GCSF concentration the diffusion coefficient decreases, probably because the effect in the light scattering produced by the protein prevails on the effect given by the cyclodextrins. Thus, the application of DLS on cyclodextrins containing formulations is questionable.

Table 6. Infinite dilution diffusion coefficient

Formulation	**D_0 (cm^2/s) $*10^{-6}$**
GCSF 10mM ABS pH 4.5	**1.12**
GCSF 10mM β-CD	**1.65**
GCSF 10mM HP-β-CD	**1.65**

It was also possible to detect the signal given by cyclodextrins in the size distribution related to the intensity of scattered light. The results concerning the size distribution are related to liquid samples, before being stressed, even though the measurements were performed on each sample after every test. These results will not be shown in order not to burden the treatment and since there is lack of coincidence with the correlation function. However, an overview about the hydrodynamic radius is presented in the figure 31.
The hydrodynamic radius of GCSF is about 2 nm; while the radius of β-CDs is about 0,8 nm. In the size distribution of the formulation with excipients it was indeed possible to notice the widening of the peak corresponding to 2 nm towards lower radius values, in presence of excipients. This point is confirmed by the size distribution obtained by the buffer, in absence of protein. Furthermore, the size distribution suggests the presence of aggregates. However, this signal is probably given by a single aggregate, because it is not detectable in the size distribution based on mass percentage instead of intensity. The samples were centrifuged before performing the measurements; thus, big aggregates were mostly removed.

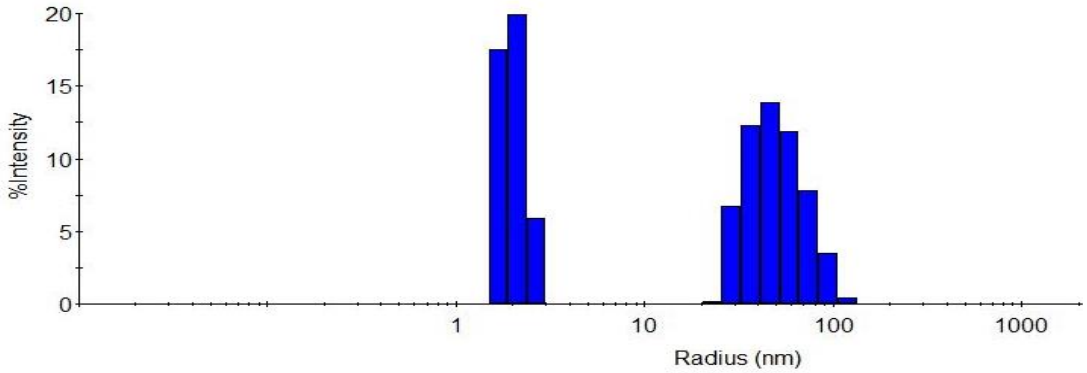


Figure 25- Size distribution of GCSF pH 4.5 based on the intensity percentage of scattered light

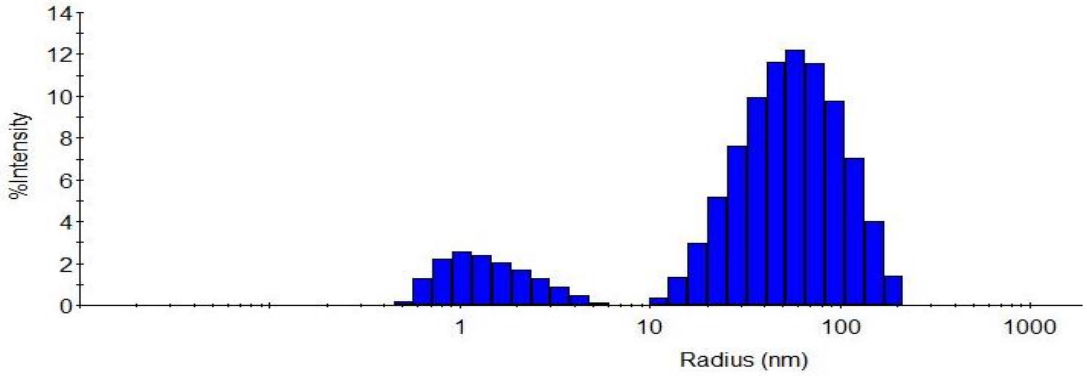


Figure 26 - Size distribution of GCSF pH 4.5 with β-CD based on the intensity percentage of scattered light

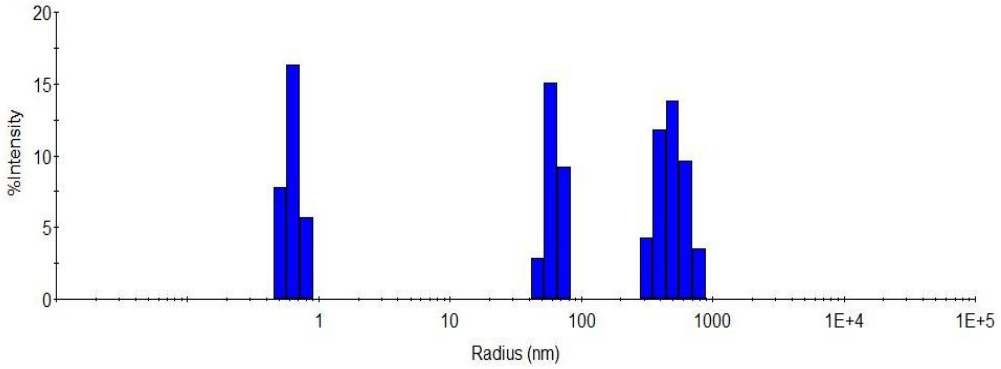


Figure 27 - Size distribution of ABS with β-CD based on the intensity percentage of scattered light

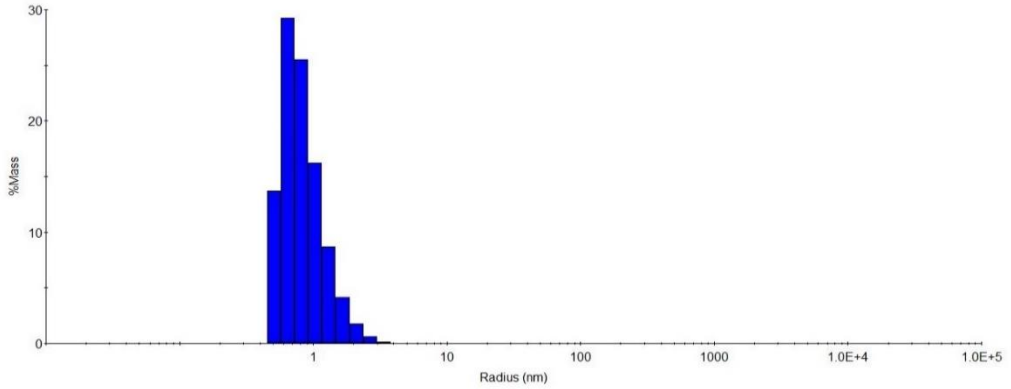


Figure 28 - Size distribution of GCSF pH 4.5 based on mass percentage

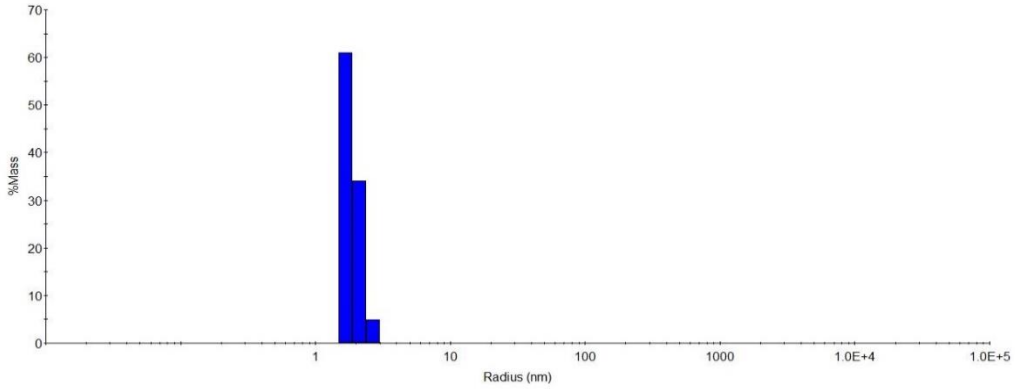


Figure 29 - Size distribution of GCSF pH 4.5 with β-CD based on mass percentage

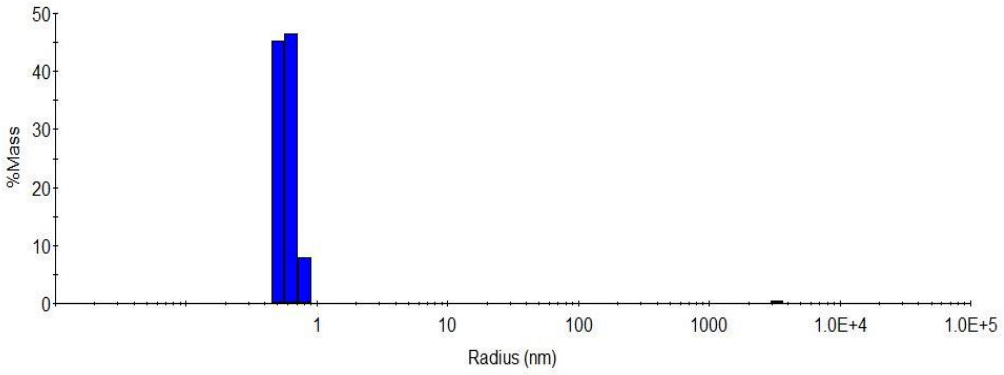


Figure 30 - Size distribution of ABS with β-CD based on mass percentage

To summarize the results of DLS, the average hydrodynamic radius is shown in the following bar-chart, that reports the radius in each formulation right after preparation and after accelerated stability tests or storage. As described earlier, the cyclodextrins have a strong effect on the scattering signal and therefore lead to a poor quality of the autocorrelation function. Consequently, the results for the hydrodynamic radius should be taken with caution. It is indeed possible to notice high values of r_h that should be related to the massive presence of aggregates in β-CD containing formulations. It is indeed possible to notice high values of r_h that should be related to the massive presence of aggregates in β-CD containing formulations.

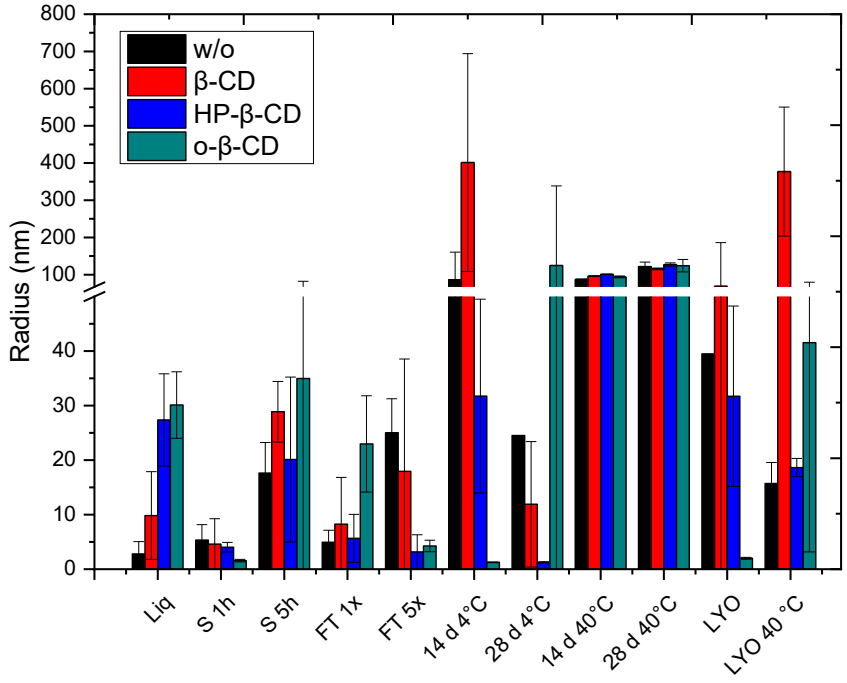


Figure 31 - Hydrodynamic radius for GCSF pH 4.5 in ABS without any excipient (black), with β-CD (red), with HP- β-CD (blue), with maltoheptaose (green)

1.2.6. Fluid Imaging Microscopy

The fluid imaging microscopy has been performed to further investigate the aggregation behaviour in the different formulations. The following bar charts present the number of particles in each formulation right after preparation and after accelerated stability tests or storage. In figure 32 It is presented the number of particles which diameter is higher than 1 μm and in figure 33 the number of particles with a diameter higher than 10 μm. These class of particles have been selected because they well represent the overall behaviour of the samples and because they allow to distinguish between excipients molecules and aggregates.

It was possible to notice the massive presence of particles with diameter lower than 1 μm in β-CD formulations, even though the sample has not been subjected to any stress. This could be related to the solubility of β-CD, close to the concentration in the samples. In fact, the number of particles was lower when the samples are stored at 40°C, while it was particularly high when they were stored at 4°C or freeze thawed. The pH shift from 4.5 to 7 in lyophilized samples should promote the dissolution of β-CD, but it promotes the aggregation of the protein [85] and the intervention of low temperatures is also relevant.

The number of particles was also high in maltoheptaose formulations, in freeze thawed, lyophilized samples and those which have been long-term stored at 40°C. Thus, it is possible to assume that the maltoheptaose is promoting the formation of particles in the samples. This is consistent with results of other studies, where maltodextrins ability as bulking agents and cryoprotectants has been investigated with MD simulations. [89][23]It is possible to assume that maltoheptaose is not interacting with proteins as much as the closed form of cyclodextrins, whereas it may self-interact, forming huge number of aggregates.

Furthermore, in the MD simulations, the propensity of β-CD (open and closed form) to form large number of clusters with small dimension has been detected, even though the open form tends to form slightly larger aggregates with respect to the closed form, while the HP-β-CD has shown propensity to form less clusters with higher dimension. [23] Thus, the presence of small particles in β-CD and maltoheptaose formulations could be explained to the formation of several clusters.

The overall trend is the presence of higher number of particles when the protein is formulated with one of the excipients. The promotion of aggregation by excipients can also be seen in the chart that shows the number of particles with a diameter of less than 10 μm. The β-CD and maltoheptaose formulation showed a huge propensity to form aggregates, while the behaviour of HP-β-CD formulations is like the samples formulated in ABS without any excipient. The highest number of particles has been detected in lyophilized samples after 28 days of storage at 40°C. It can be assumed that the natural propensity to aggregation of GCSF has been enhanced by the formation of the ice-water interface and by the drying occurred during lyophilization. Then the cake structure was not maintained at high temperature, so GCSF was not protected from the heat stress. Furthermore, in literature it is possible to find that the percentage

of insoluble aggregates increases over time, during incubation at 37°C, when the protein concentration is 1 mg/mL. [85]

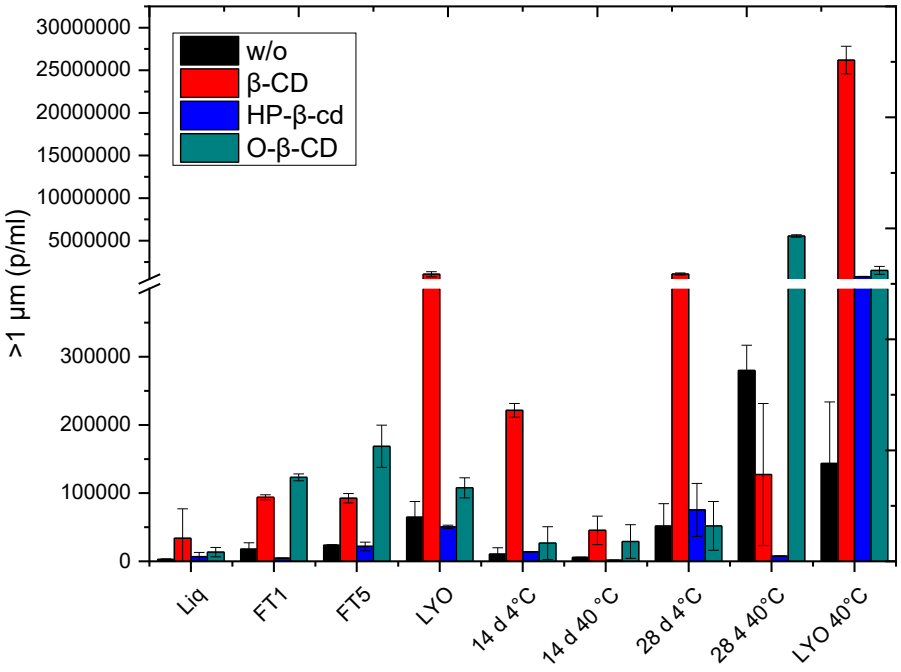


Figure 32 - Number of particles with diameter higher than 1 µm for GCSF pH 4.5 in ABS without any excipient (black), with β-CD (red), with HP- β-CD (blue), with maltoheptaose (green)

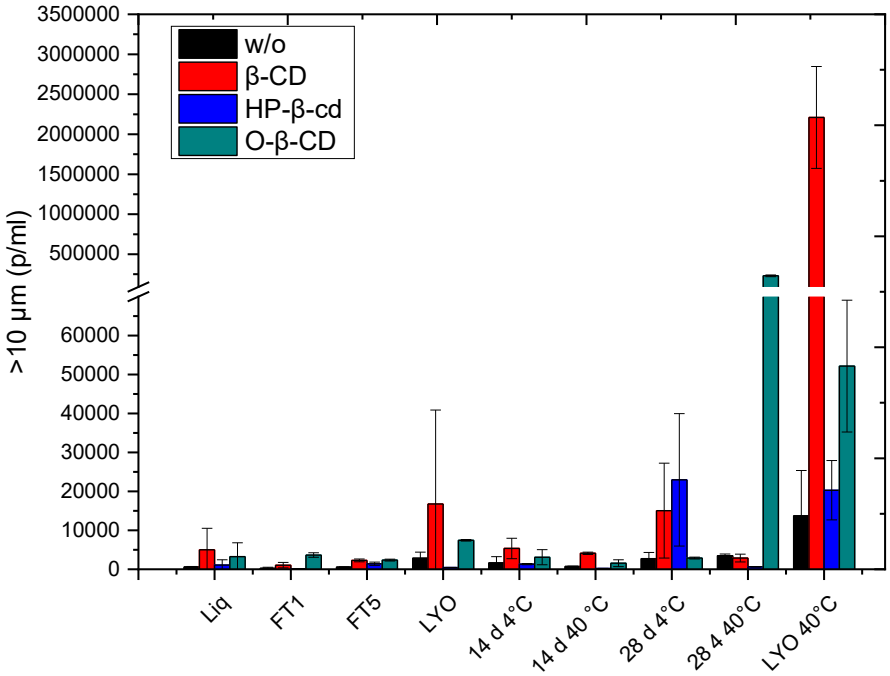


Figure 33 - Number of particles with diameter higher than 10 µm for GCSF pH 4.5 in ABS without any excipient (black), with β-CD (red), with HP- β-CD (blue), with maltoheptaose (green)

1.3. Monomer recovery

The evaluation of monomer recovery is the quantification of the amount of protein left in each sample after stress or storage at different temperature compared to the content of the liquid samples right after formulation. It is a way to quantify the activity of formulations, assuming that the left monomers are active. It is presented as bar chart where it is possible to read the value in each formulation right after preparation and either after accelerated stability tests or storage (fig. 34)

The protein was almost completely recovered after shaking, freeze-thawing and storage at 4°C, except for the maltoheptaose formulation, which seems to be the most sensitive to the extended shaking. The storage at high temperature and the lyophilization affected the recovery of the protein more distinct; in fact, the protein content is lower.

Looking at the fluid imaging microscopy results, it is reasonable to assume that part of the protein formed aggregates during lyophilization and long term storage of liquid and lyophilized samples at 40°C and it has been removed by centrifugation of samples performed before putting them into the RP-HPLC system.

From this overview, it is possible to notice that the addition of excipients is not significantly improving the preservation of protein activity.

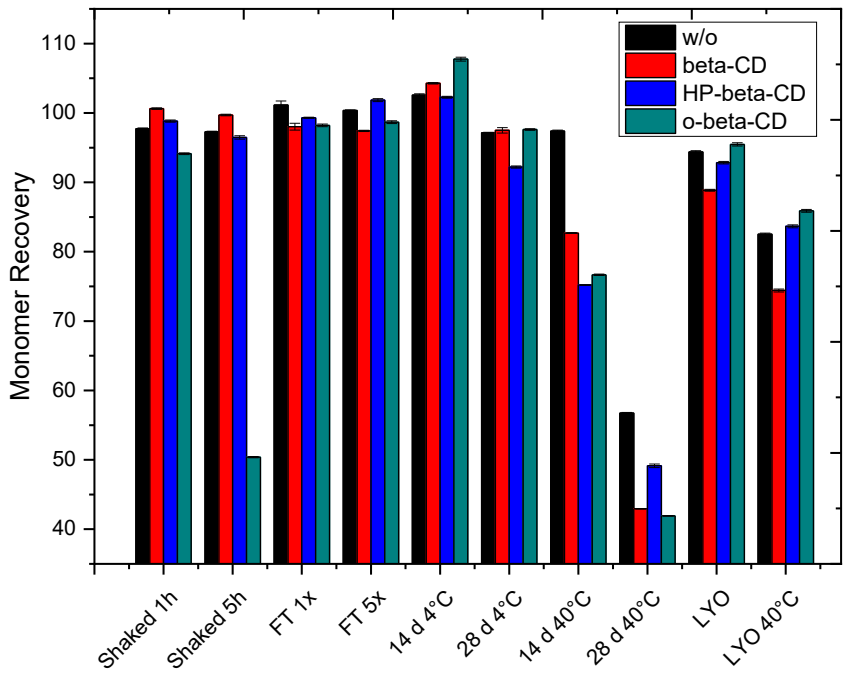


Figure 34 - Monomer recovery percentage after accelerated stability test and storage of GCSF pH 4.5 in ABS without any excipient (black), with β-CD (red), with HP- β-CD (blue), with maltoheptaose (green)

Furthermore, it is important to highlight the presence of a peak corresponding to hydrophilic species in samples formulated with HP-β-CD when they are stored at 4°C (14 and 28 days), after the 2 weeks of storage at 40°C and after lyophilization. This could mean that chemical degradation, like oxidation occurred. After 28 days of storage at 40°C of liquid and lyophilized samples containing the excipient it was possible to notice the presence of peaks correspondent to hydrophobic and hydrophilic species. Peaks attributable to the presence of hydrophobic species were also detectable in all other formulations stored at 40°C after lyophilization. The presence of hydrophobic species may be related to the exposure of hydrophobic residues of the protein. When the native form of the protein is preserved the hydrophobic side chains are usually buried in the core of the structure.[90]
Secondary peaks are considered relevant when their area occupies more than 1% of the overall area. Some chromatograms are shown as examples.

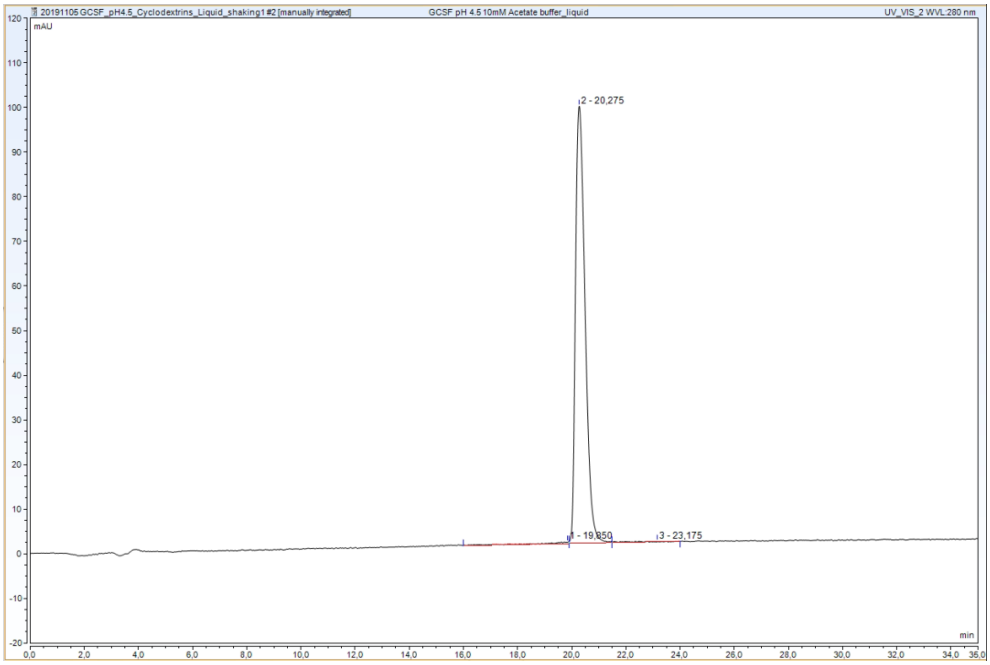


Figure 35 - Chromatogram of GCSF in ABS pH 4.5

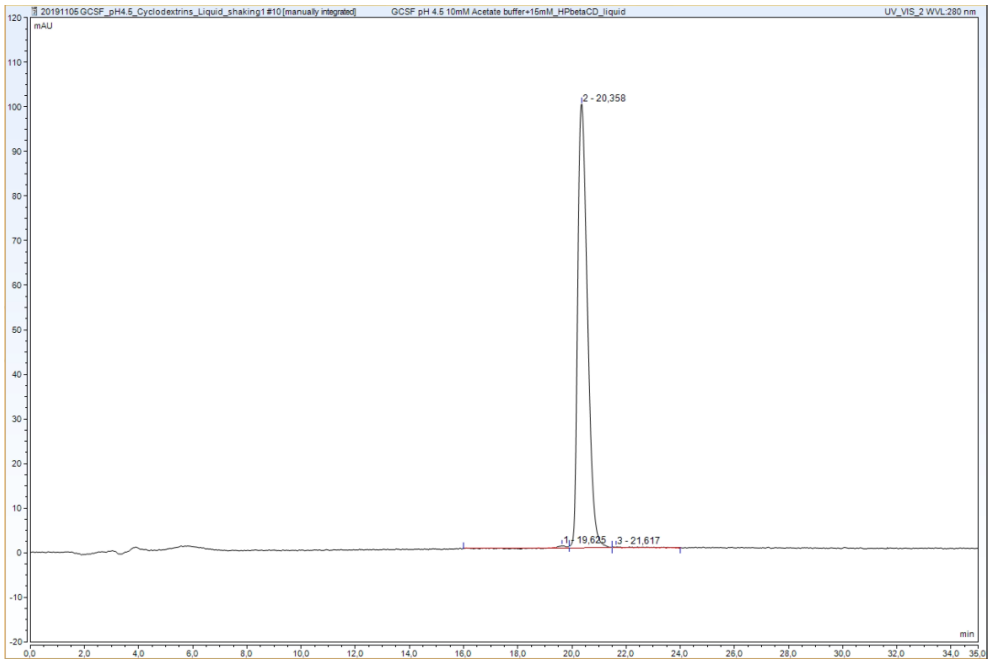


Figure 36 - Chromatogram of GCSF in ABS pH 4.5 with HP-β-CD

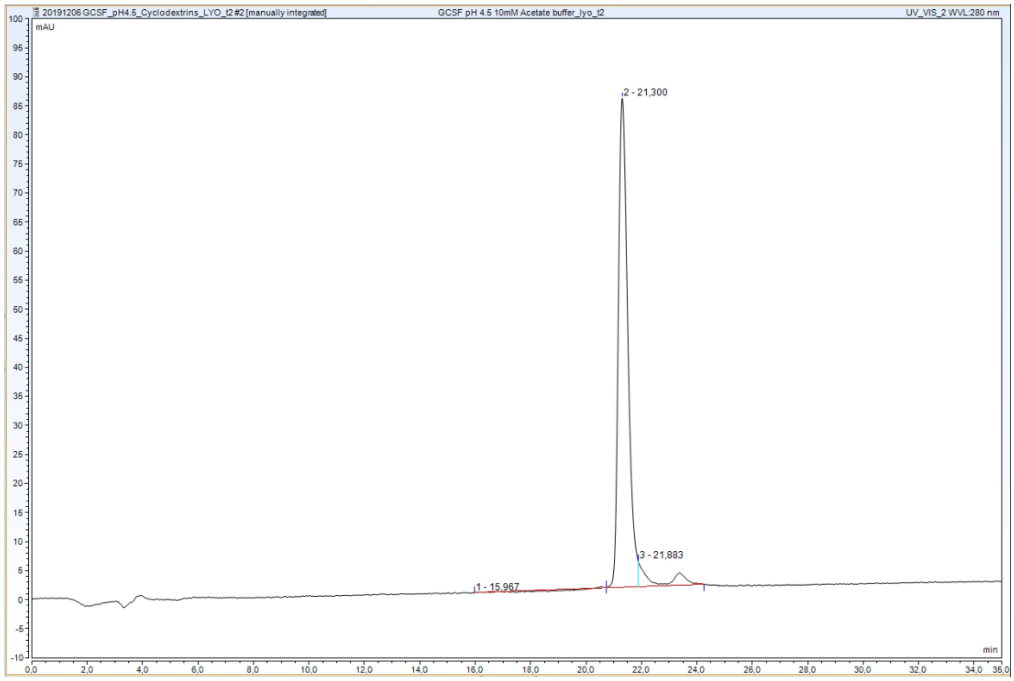


Figure 37 - Chromatogram of GCSF in ABS pH 4.5 after lyophilization and storage for 28 days at 40°C

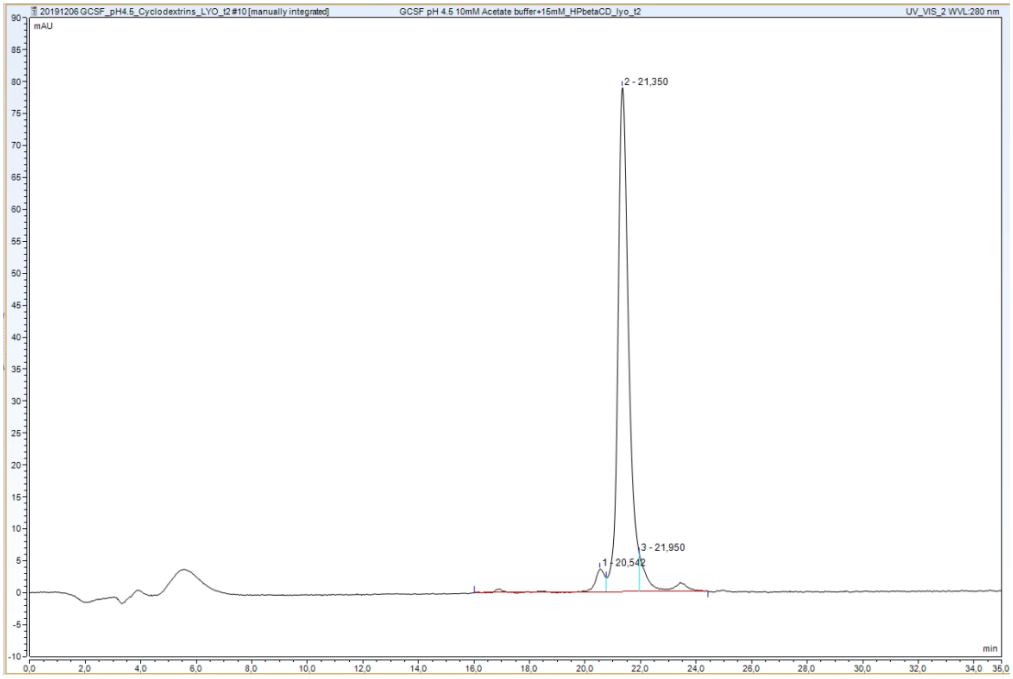


Figure 38 - Chromatogram of GCSF in ABS pH 4.5 with HP-β-CD after lyophilization and storage for 28 days at 40°C

1.4. Visual appearance of lyophilized samples

The structure of lyophilized sample appeared collapsed when the protein was formulated without any excipient and with β-CD, while the lyophilized cake was more elegant and stable when HP-β-CD and maltoheptaose have been used as excipients. The visual appearance of the samples did not change after storage.

The first sample on the left of figure 39 was collapsed because no lyoprotectant or bulking agent was present, while the second one is collapsed as well because β-CD did not act as bulking agent.

Concerning maltoheptaose, is well known that sugars are widely used in biopharmaceutics as lyoprotectants and bulking agents, [91] even though high molecular weight maltodextrins are not that effective at protecting protein structure in drying phase.[89][92]

HP-β-CD has been documented in literature as good lyoprotectant for different proteins.[93][94]

Figure 39 - Lyophilized samples of GCSF

2. LDH

2.1. Binding of the cyclodextrins to the protein

The binding of cyclodextrins with LDH has been investigated with HP-β-CD, only because loading of LDH on sensors did not give a satisfying signal. Thus, there was no binding signal of the excipient. The loading was also insufficient when the protein concentration was increased, and the immobilization step lasted longer. The device is probably not suited for this protein or the biotinylating did not work properly with the LDH and it is necessary to try other methods to investigate the binding of cyclodextrins and maltoheptaose to LDH. It would have been useful to investigate the binding of β-CD, because, according to simulations[23], it should interact the most with the protein.

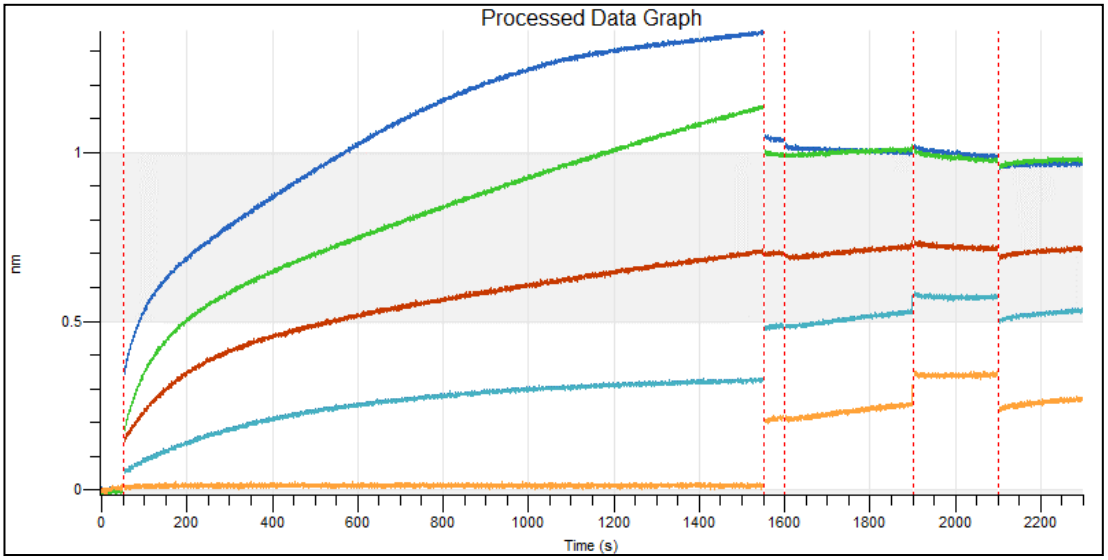


Figure 40 - Biolayer thickness as function of time during the loading test of LDH pH 7.0 with concentration 0.06, 0.02, 0.0067 g/L using HP-β-CD 20 mM as ligand

2.2. Protein aggregation and conformational stability

2.2.1. pH

The pH has been measured in each sample before and after stresses or storage. The value detected was 7±0.09 and it has not significantly changed.

2.2.2. Circular Dichroism

Circular dichroism has been used to detect eventual changes in LDH tertiary structure, induced by cyclodextrins or maltoheptaose. The spectra of the formulations investigated in the near-UV region are shown in figure 41. It was possible to appreciate the see in tertiary structure induced by excipients, but it was not possible to distinguish

between the behaviour of maltoheptaose and that of cyclodextrins. The presence of the shift but being the shape of the spectra unaltered suggests that the environment of aromatic residues is unchanged.

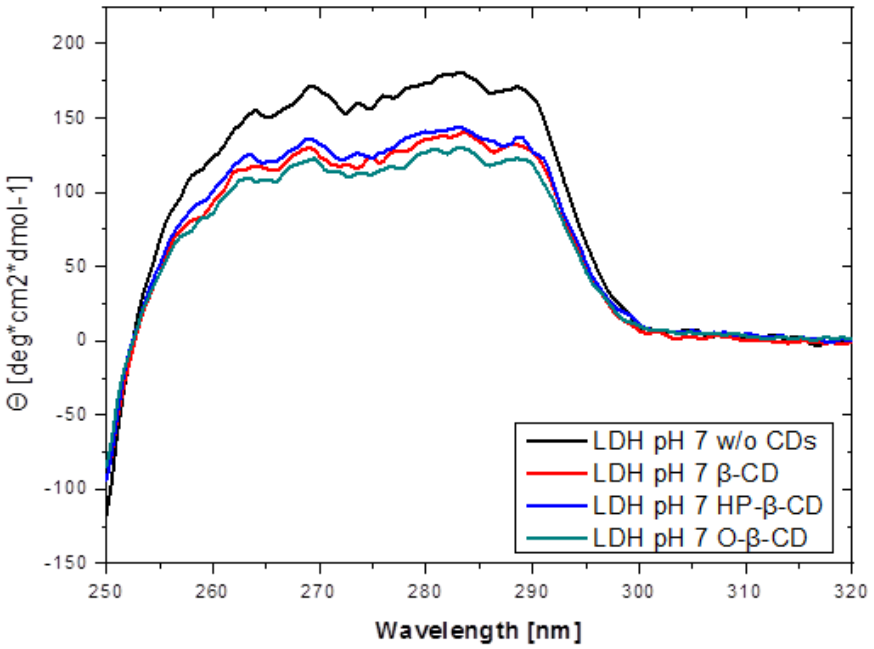


Figure 41 - CD spectrum in the near-UV region: molar ellipticity as function of wavelength for LDH pH 7.0 in PPB without any excipient (black), with β-CD (red), with HP- β-CD (blue), with maltoheptaose (green)

2.2.3. NanoDSF

Thermal stability of the protein has been investigated with nanoDSF, in the temperature range 20-90°C. In figure 42 it is possible to see the ratio of integrated fluorescence, which allows to detect the temperature of unfolding. There was no considerable difference between the formulations. Figure 43 shows the scattering values as function of temperature and gives the aggregation onset temperature value. It was possible to notice a slight shift in absorbance within 60-90°C when the protein was not formulated with any excipient. T_{agg} is about 52,5°C and T_m is about 58°C. The difference in T_{agg} between different formulations lied within the error bars, calculated as standard deviation between triplicates, and the difference in T_m are less than 0.5°C.

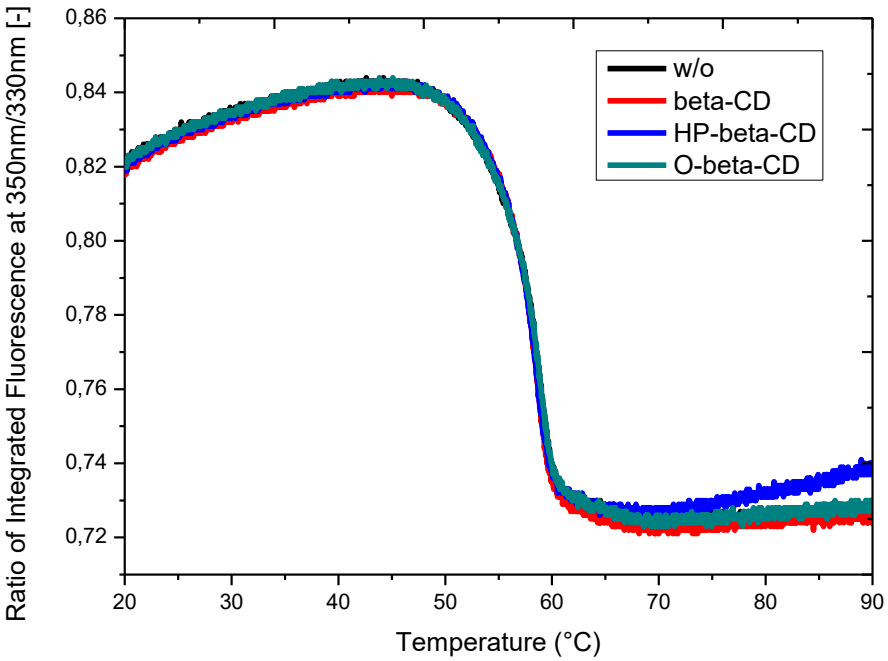


Figure 42 - Ratio of integrated fluorescence at 350nm/330nm as function of temperature for LDH pH 7.0 in PPB without any excipient (black), with β-CD (red), with HP- β-CD (blue), with maltoheptaose (green)

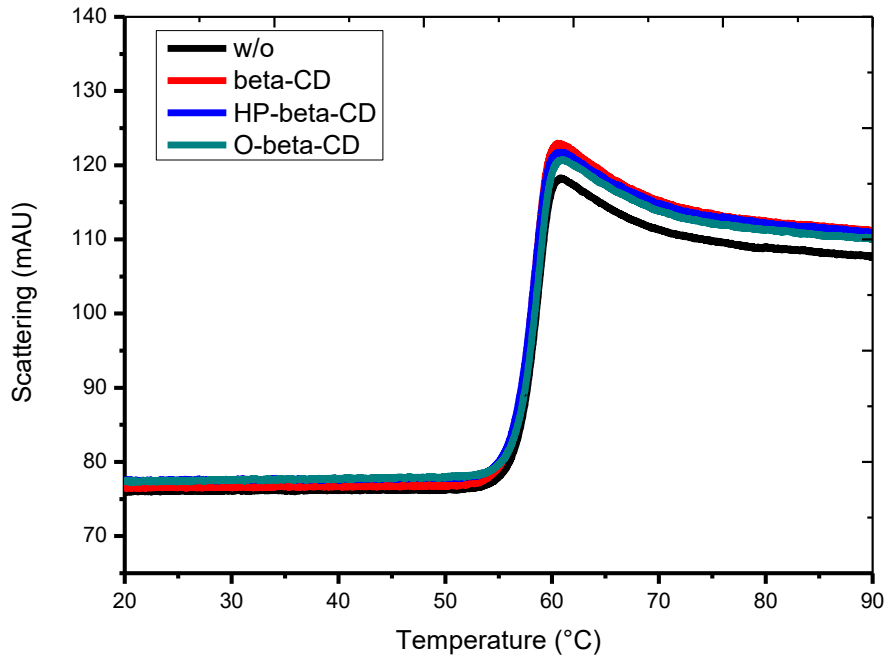


Figure 43 - Scattering intensity as function of temperature for LDH pH 7.0 in PBS without any excipient (black), with β-CD (red), with HP- β-CD (blue), with maltoheptaose (green)

Table 7. Aggregation temperature and melting temperature of LDH formulations

Formulation	**Tagg**	**Err**	**Tm**	**Err**
LDH 10 mM PBS pH 4.5	52,42	0,31	58,16	0,02
LDH 10 mM β-CD	52,35	0,44	57,92	0,01
LDH 10 mM HP-β-CD	52,84	0,27	57,86	0,04
LDH 10 mM O-β-CD	52,60	0,60	58,14	0,04

2.2.4. DLS

The dynamic light scattering is useful to investigate the size distribution of particles in the samples. It is presented in terms of percentage intensity of scattered light and in terms of mass. The following figures show the distribution in liquid samples, before exposure to stresses, when the protein was formulated without any excipient and with β-CD, as examples. These results are useful to highlight that the excipients are detectable by light scattering because they caused a widening of the peak correspondent to the protein toward lower radius values, while the intensity was lower. In fact, the hydrodynamic radius of LDH is 4 nm, while the β-CDs radius, as previously said, is about 0.8 nm. This is confirmed by the size distribution elaborated for the buffer with β-CD, in absence of protein.
Furthermore, in size distributions based on percentage intensity of scattered light it was possible to notice the presence of peaks correspondent to higher values of radius (e.g. 100 nm), while there were no aggregates detectable in the distributions in terms of mass, in the figures shown.

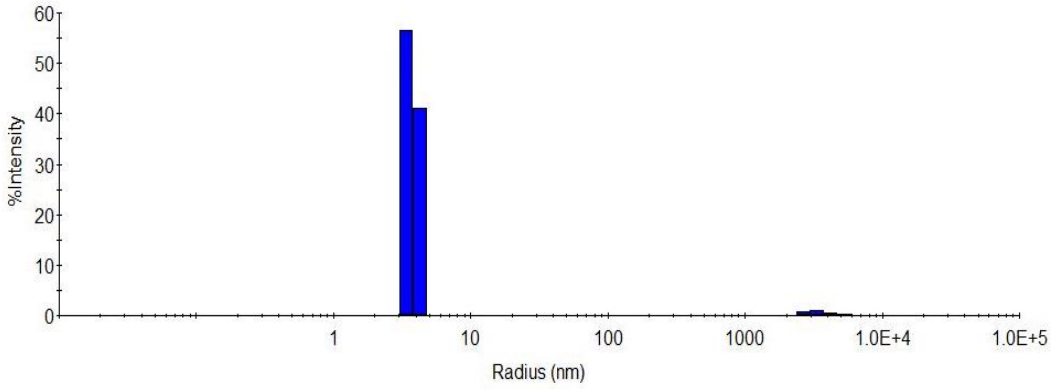


Figure 44 - Size distribution of LDH pH 7.0 based on the intensity percentage of scattered light

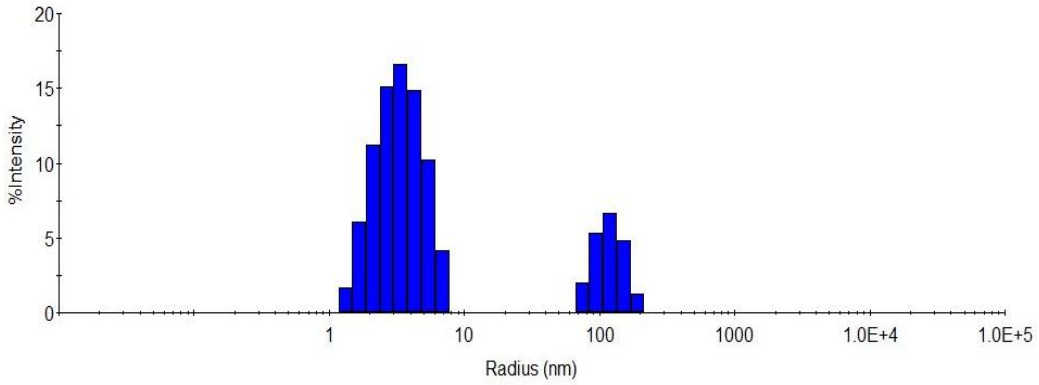


Figure 45 - Size distribution of LDH pH 7.0 with β-CD based on the intensity percentage of scattered light

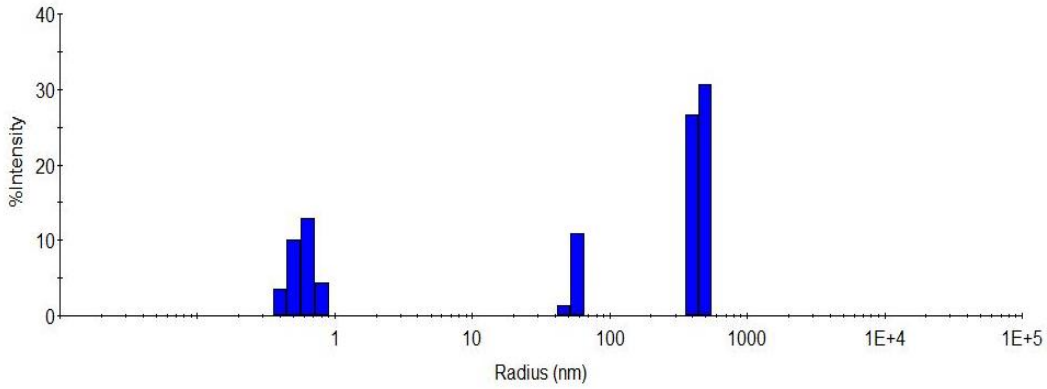


Figure 46 - Size distribution of PPB with β-CD based on the intensity percentage of scattered light

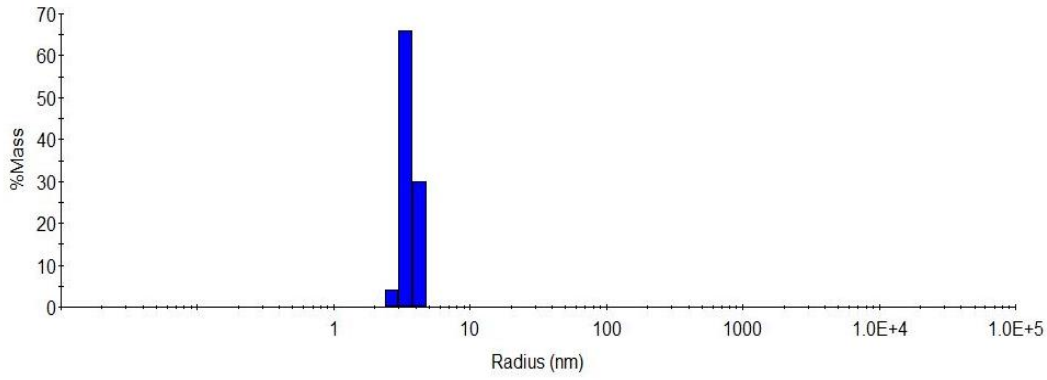


Figure 47 - Size distribution of LDH pH 7.0 based on mass percentage

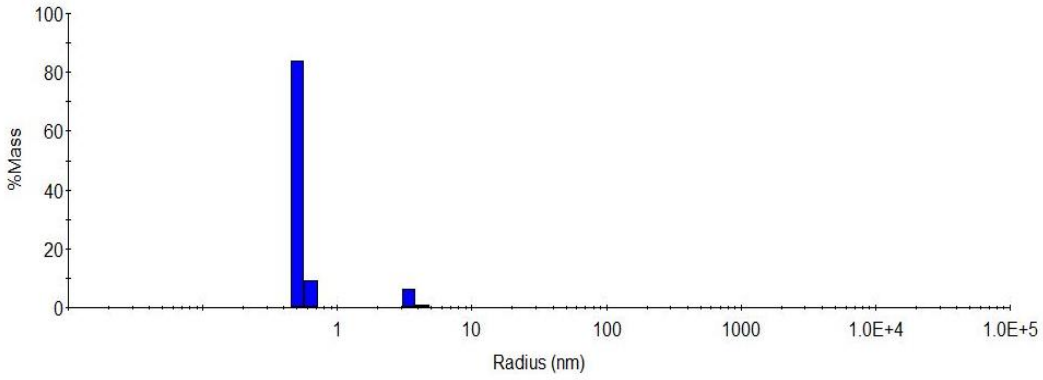


Figure 48 - Size distribution of LDH pH 7.0 with β-CD based on mass percentage

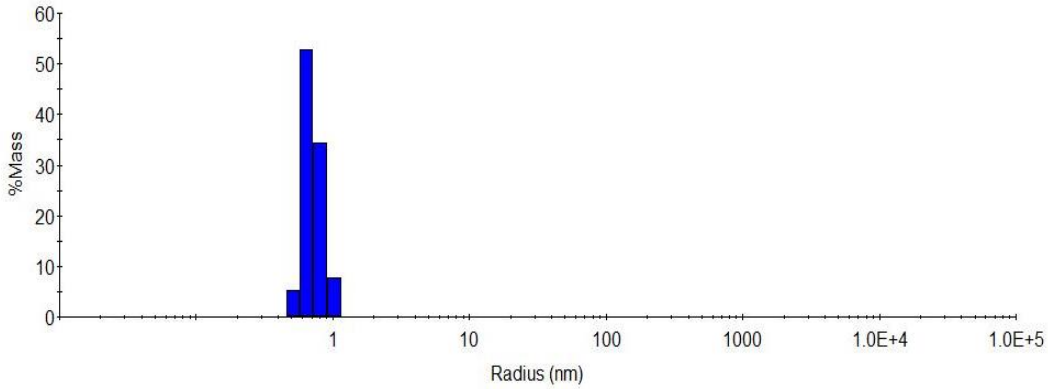


Figure 49 - Size distribution of PPB pH 7.0 with β-CD based on mass percentage

The bar charts showing the values of hydrodynamic radius (fig. 50) and percentage polydispersity (fig. 51) summarize the results collected by DLS, in each formulation right after preparation and after accelerated stability tests or storage. The radius hydrodynamic in formulations without any excipient was 4 nm, whereas is a slightly lower when the excipients are present but close to the value of the protein. Samples stored at 40°C for 28 days, lyophilized samples and lyophilized samples stored at 40°C showed different behaviour and they also showed the highest PD% values, which suggested the presence of particles of different sizes. In fact, the PD% values for those samples correspond to polydisperse system. It is also important to notice that the polydispersity is lower when the protein is not formulated with any excipient, while is higher when excipients are present, because they are smaller, and the samples also contain aggregates.

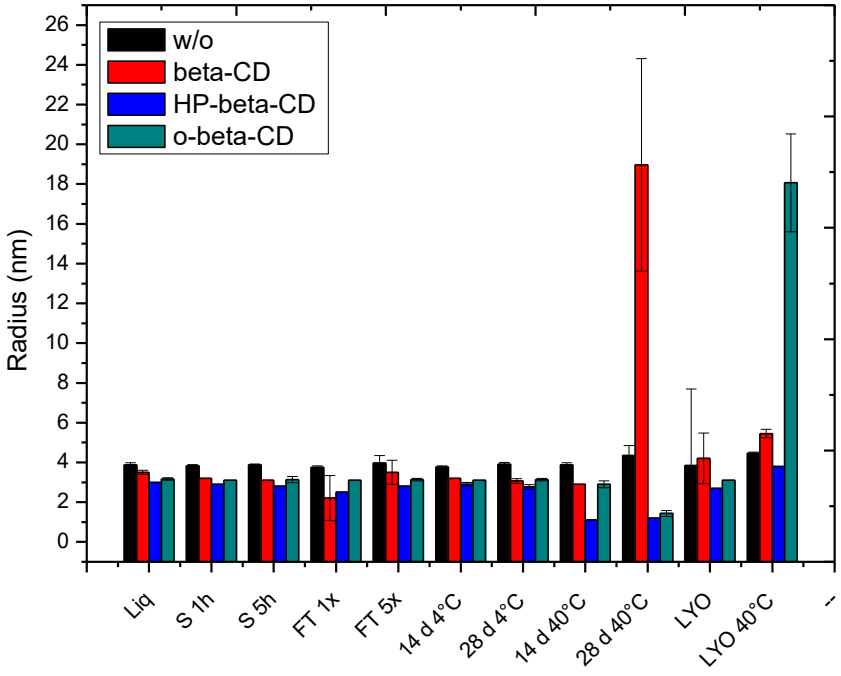


Figure 50 - Hydrodynamic radius for LDH pH 7.0 in PPB without any excipient (black), with β-CD (red), with HP- β-CD (blue), with maltoheptaose (green)

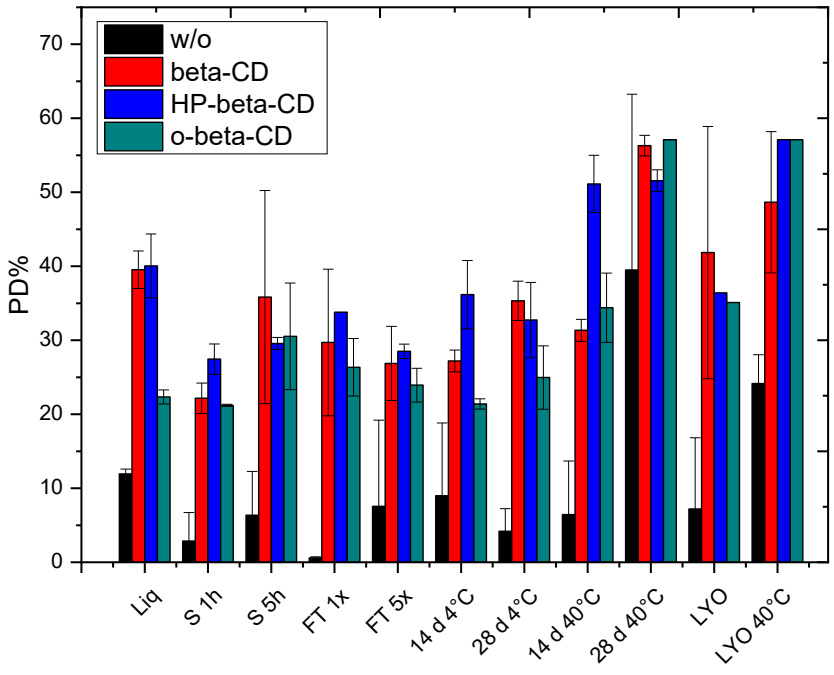


Figure 51 – Polydispersity index percentage for LDH pH 7.0 in PPB without any excipient (black), with β-CD (red), with HP- β-CD (blue), with maltoheptaose (green)

2.2.5. Fluid Imaging Microscopy

The fluid imaging microscopy, as previously discussed, is useful to investigate the presence of particles in the samples and to classify them on the basis of size. The results showed are related to the number of particles which diameter is higher than 1 μm (fig. 52) and higher than 10 μm (fig 53).
In shaken samples, samples subjected to a single cycle of freeze-thawing and samples stored at 4°C and 40°C the particles number was moderate, and close to the amount detected in liquid samples measured right after formulation. The number of particles was higher in samples subjected to five cycles of freeze-thawing, lyophilized samples and samples stored for 28 days at 40°C after freeze-drying. In particular, in lyophilized samples, the particles amount is deeply high in β-CD (open and closed form) formulations.
However, it is important to highlight that in liquid samples stored at 40°C a thin transparent layer (fig. 54) was found on the top of samples, probably related to the aggregation of the protein itself; thus, the amount of protein and, consequently, of particles detected is low. Since the samples have not been filled aseptically, microbial growth might also be a reason for the layer formation.
The formulations without any excipient showed the best overall behaviour and samples where HP-β-CD is used as excipient contain similar number of particles in almost all cases.
The MD simulations suggested the ability of β-CD to protect the protein from aggregation, because it should be the most included by the protein itself [23], while DLS and fluid imaging microscopy results showed the opposite trend. For the HP-β-CD, the MD simulations highlighted the preferential exclusion of the excipient as stabilizing mechanism: HP-β-CD could be a good lyoprotectant.[23] In this case, the experimental results seem to match with simulation results.

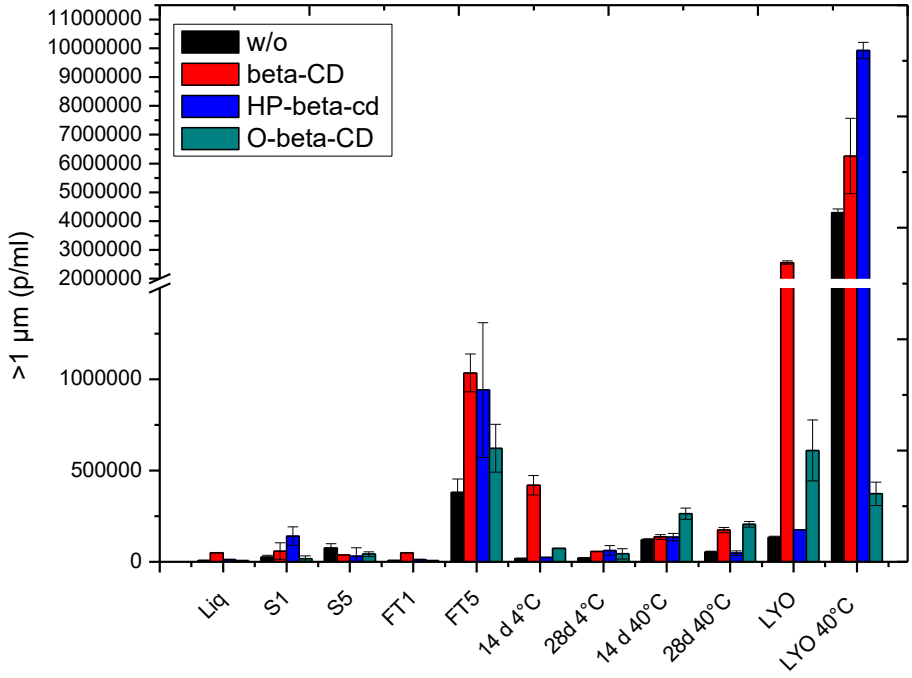


Figure 52 - Number of particles with diameter higher than 1 µm for LDH pH 7.0 in PPB without any excipient (black), with β-CD (red), with HP- β-CD (blue), with maltoheptaose (green)

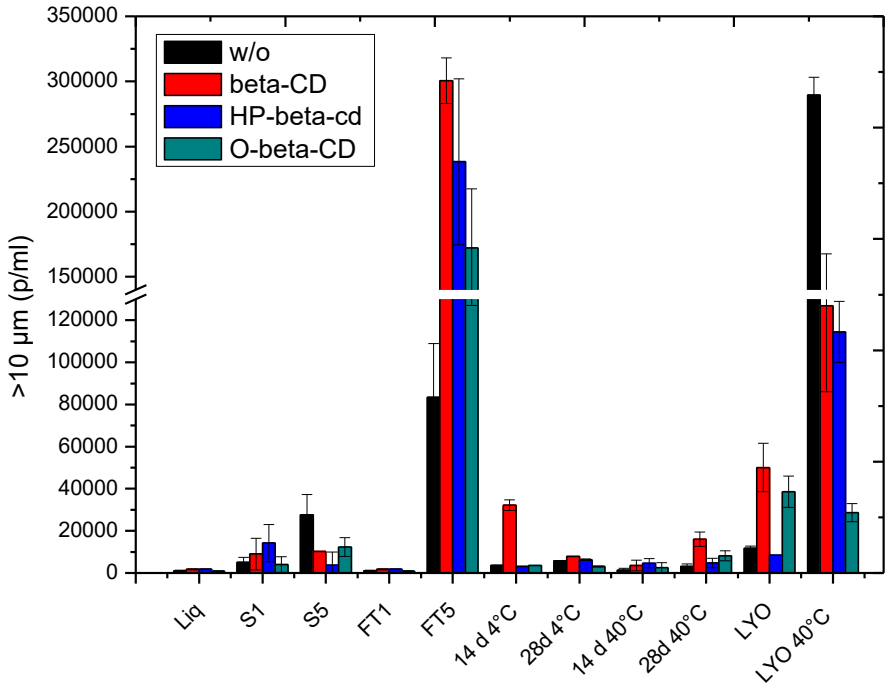


Figure 53 - Number of particles with diameter higher than 10 µm for LDH pH 7.0 in PPB without any excipient (black), with β-CD (red), with HP- β-CD (blue), with maltoheptaose (green)

Figure 54 - LDH samples after 14 days of storage at 40°C

2.3. Activity assay

The activity assay is useful to measure the activity as absorbance increase, which is related to the amount of NADH produced in each sample, within the linear range of the standard curve. The activity of the protein has been investigated in pre-tests, in order to find the proper concentration to reach an absorbance within the linear range of the standard curve The first concentration range tested has been based on what has been reported in literature.[95] It is important to remind that the concentration chosen is 0.1 µg/mL, even though the results of these pre-tests will not be shown, to do not burden the treatment of the topic.
Furthermore, the activity of LDH in stock since 2016 has been compared with the freshly purchased batch. The fig. 55 shows the activity values in nmol NADH/well. They are within the range of the linear fit of the standard; while in the table the activity values are reported.

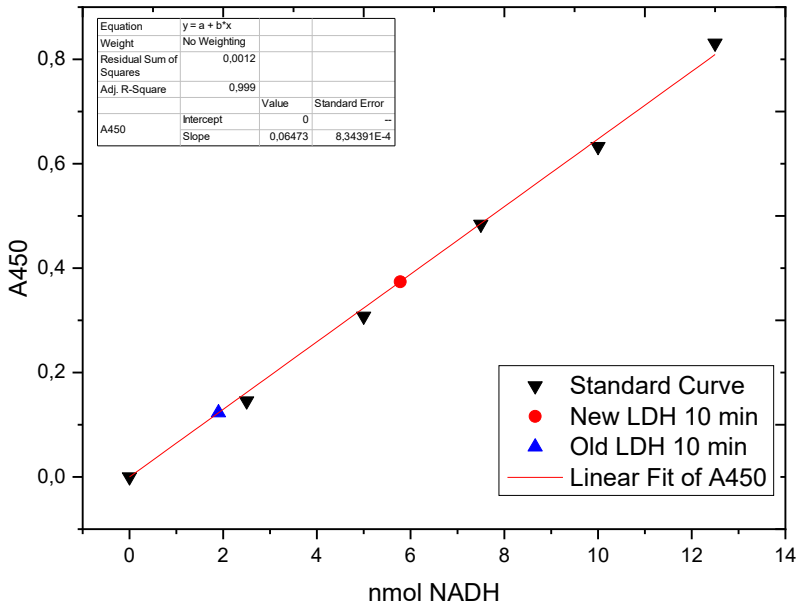


Figure 55 - Comparative test of LDH activity: Absorbance at 450 nm as function of nmol of NADH formed. Activity of the protein in stock since 2016 (blue triangle) is compared to the activity of the fresh one (red dot); standard curve (red line) built as linear fit of the experimental datapoint (black triangle)

Table 8. LDH activity in the comparative pre-test

Protein	**Activity**	**Err**
LDH 2016	15,68	0,11
LDH 2019	41,17	1,20

The activity results, it was possible to notice that the activity is considerably higher in formulations containing HP-β-CD, except in samples stored at 40°C. However, as previously stated, the protein probably aggregated, forming a layer on the top of the sample, thus the activity is lower.
In shaken samples the activity did not show a significant difference with respect to the values in unstressed samples.
The activity was about two times higher in each formulation, when the samples were freeze-thawed, freeze-dried and long term stored at 4°C.
To further investigate the behaviour of formulations at low temperatures, the activity freeze-thawed samples has been measured after 3 days of storage at 4°C and it was lower, probably because the initial conformation was restored.
The significant activity increase has been a surprising result, because for LDH from rabbit muscle the optimal conditions are pH 7.0, 45-55°C.[100] However it has been previously documented: LDH isozyme activity after lyophilization increased up to fourfold, even though in the case of LDH from rabbit muscle the effect was weaker than for LDH from porcine heart.[98] Since LDH can exist in different catalytic active conformations, and they showed different activities,[98][101] it is possible to assume that not-denaturing stresses promote the conformational change,[98] which is related to the activity increase.
The ability of HP-β-CD to protect LDH activity from damage during freeze-thawing and freeze-drying has been previously reported [96][15] and it has also been shown that HP-β-CD is the best lyoprotectant, compared to other CDs.[97] Other studies found that HP-β-CD does not promote the damage, induced by freezing but does not preserve the quaternary structure of the protein.[98]
β-CD showed a good preservation of the initial activity increase, in literature it is possible to find that it is not as much effective as the hydroxy propylated form.[97]
Furthermore, in almost each study documented of LDH activity in presence of cyclodextrins the initial concentration of the protein was lower than 0.5 mg/mL, thus it is possible to speculate about the self-protection of the protein against the denaturation induced by the stresses performed on samples. Therefore, the activity of LDH has been further investigated at different concentration.

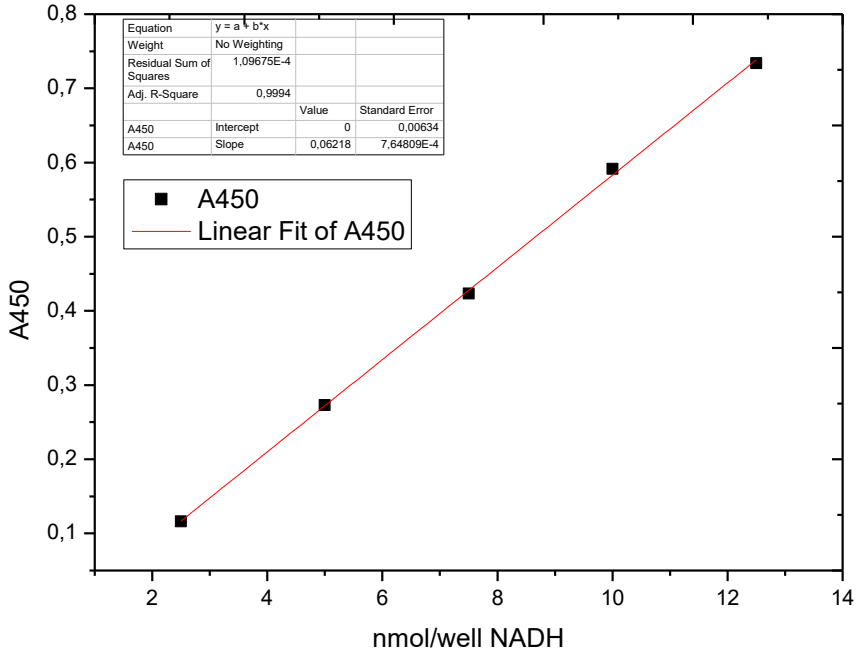


Figure 56 – Absorbance at 450 nm as function of nmol/well of NADH formed: standard curve (red line) built as linear fit of the experimental datapoint (black square)

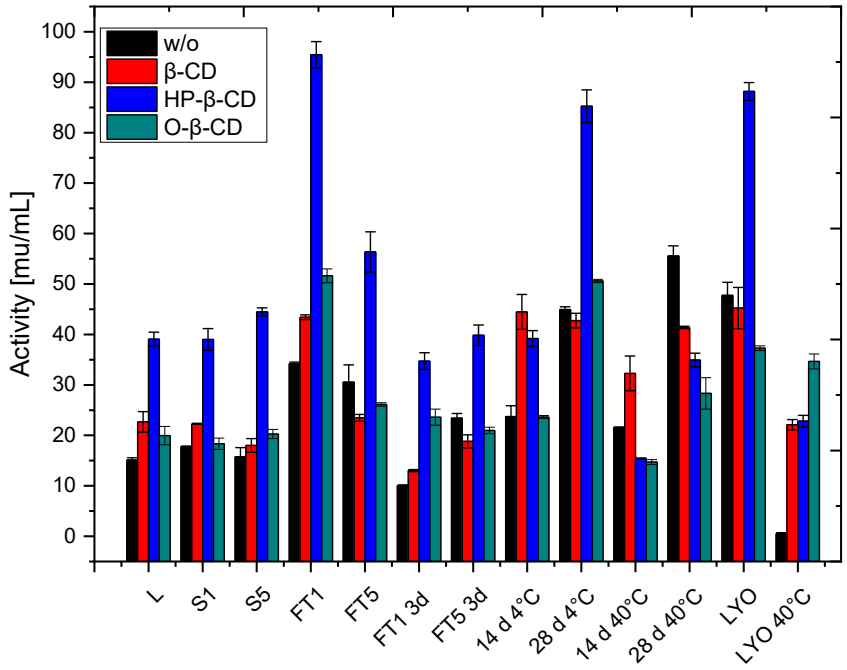


Figure 57 – Activity per mL for LDH pH 7.0 in PPB without any excipient (black), with β-CD (red), with HP- β-CD (blue), with maltoheptaose (green)

Fig. 58 and fig. 59 depict the results obtained by the activity assay performed on LDH with a concentration of 0.005 mg/mL, 100 times lower than in the previous set of experiments. Here, the activity of the protein was measured for formulations with sucrose, a good cryoprotectant, and formulations with NaCl, which should promote the denaturation of the protein, being a chaotropic agent that has proved to reduce LDH activity, when the protein was subjected to freeze-thawing cycles. [33] The activity in the liquid samples after preparation is equal to the activity detected in the previous set of experiments and the value in the HP-β-CD formulation is again dramatically higher than in other formulations. In shaken samples, activity loss occurred for all samples except for the one with HP-β-CD, which behaves as a surfactant, accumulating at the air-water interface, perhaps protecting the protein form surface-induced denaturation. The activity slightly increased after freeze-thawing when HP-β-CD is present; it was constant when maltoheptaose or sucrose are used as excipients, while decreased in formulations without any excipient or with β-CD. After lyophilization the activity increased in formulations without any excipient, with HP-β-CD and sucrose. The difference was not as pronounced as in the previous set of experiments but confirmed the identified behaviour. The ability of sucrose to prevent protein dissociation, preserving the activity is confirmed in the literature,[98] although it has been previously reported to be less effective than HP-β-CD with LDH subjected to freeze drying.[15] NaCl did not affected the protein activity as much as expected, probably because the concentration of the compound was not sufficient to cause complete denaturation.

Finally, the trend is confirmed by the activity assay performed on samples after centrifugation (10 minutes at 10000 rpm), to remove aggregates, which could interfere with the absorbance measurement due to light scattering. The activity is slightly higher after centrifugation. Therefore, it is possible to assume that the presence of aggregates caused difference in light scattering, which led to lower absorbance values.

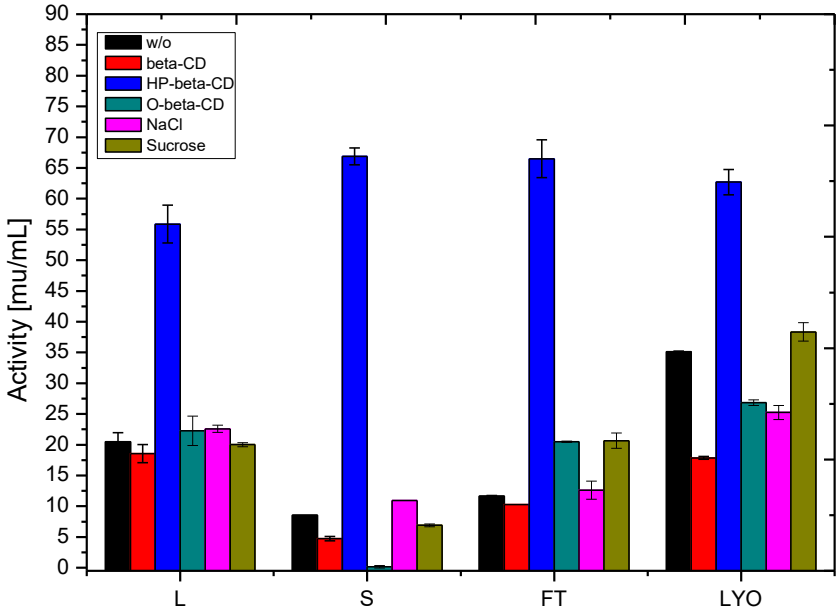


Figure 58 - Activity per mL for LDH pH 7.0 in PPB without any excipient (black), with β-CD (red), with HP- β-CD (blue), with maltoheptaose (green), with NaCl (pink), with sucrose (olive green)

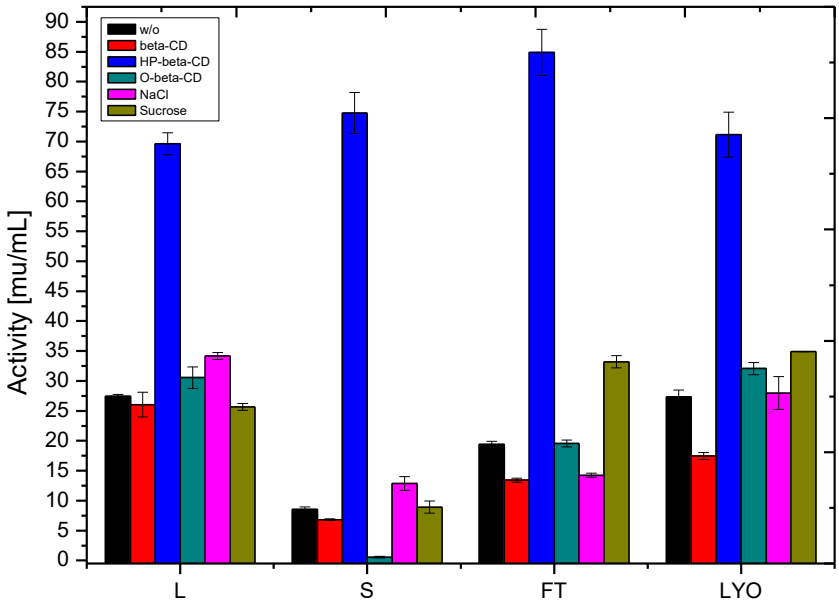


Figure 59 - Activity per mL after centrifugation for LDH pH 7.0 in PPB without any excipient (black), with β-CD (red), with HP- β-CD (blue), with maltoheptaose (green), with NaCl (pink), with sucrose (olive green)

2.4. Visual appearance of lyophilized samples

The samples structure appeared collapsed when the protein was formulated without any excipient, while the cake is more stable when the excipients are present. These results are consistent with that has been found in literature, except for β-CD (the Author is not aware of any study about the use of this excipient as lyoprotectant). Furthermore, MD simulations indicated that CDs tend to accumulate in bulk, in presence of ice-water interface, acting as bulking agents.[23] The visual appearance of lyophilized cakes was unchanged after storage at 40°C.

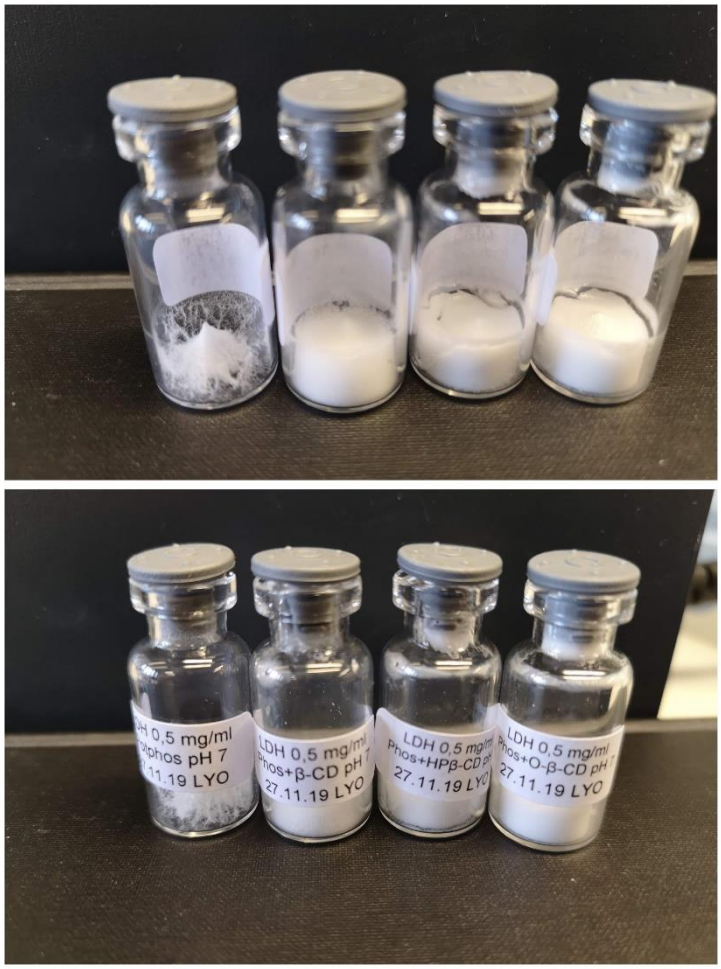


Figure 60- Lyophilized samples of LDH

3. β-CD

3.1. Fluid Imaging Microscopy

The contribution of β-CD and maltoheptaose to the huge number of particles found in GCSF formulations is confirmed by the measurements performed on ABS with the excipients at 4°C and 40°C. It is possible to see that the number of particles was relevant when the buffer contains β-CD (open and closed form), while it was low when HP-β-CD is added, or no excipients are present, and significantly reduced when the temperature increases. Furthermore, it is relevant that most particles were in the size range attributable to CD diameter.

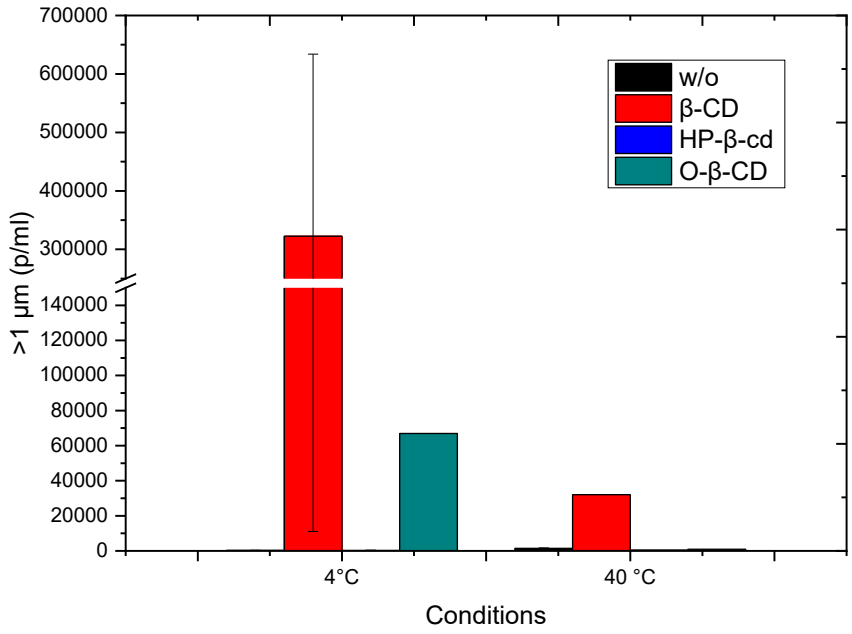


Figure 61- Number of particles with diameter higher than 1 µm for ABS at pH 4.5 without any excipient (black), with β-CD (red), with HP- β-CD (blue), with maltoheptaose (green;) stored at 4°C (first group) and 40°C (second group)

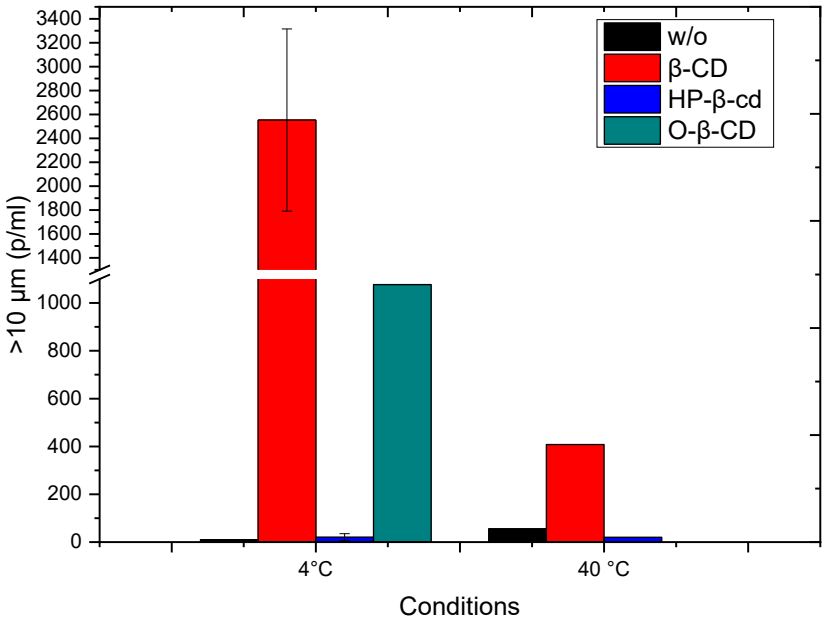


Figure 62 - Number of particles with diameter higher than 10 µm for ABS at pH 4.5 without any excipient (black), with β-CD (red), with HP- β-CD (blue), with maltoheptaose (green;) stored at 4°C (first group) and 40°C (second group)

The analysis of particles number has also been performed on PPB at pH 7.0, in presence of excipients, as well. The particles number is quite high when β-CD is present, but still lower than in the buffer at pH 4.5. The amount may be related to β-CD solubility, because it is reduced when the buffer is stored at 40°C. HP-β-CD showed the lowest particles number, because its solubility is higher, thanks to the presence of hydroxyl groups.

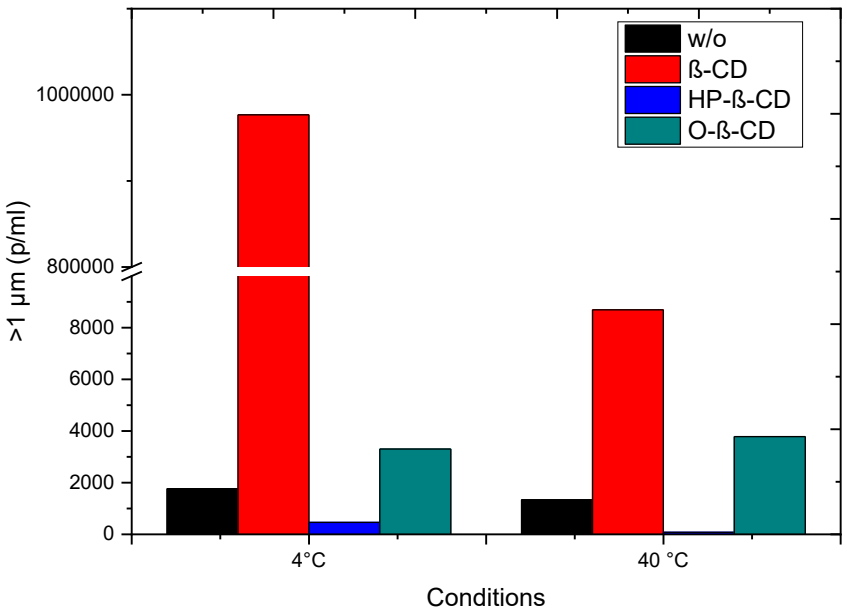


Figure 63 - Number of particles with diameter higher than 10 µm for PPB at pH 7.0 without any excipient (black), with β-CD (red), with HP- β-CD (blue), with maltoheptaose (green;) stored at 4°C (first group) and 40°C (second group)

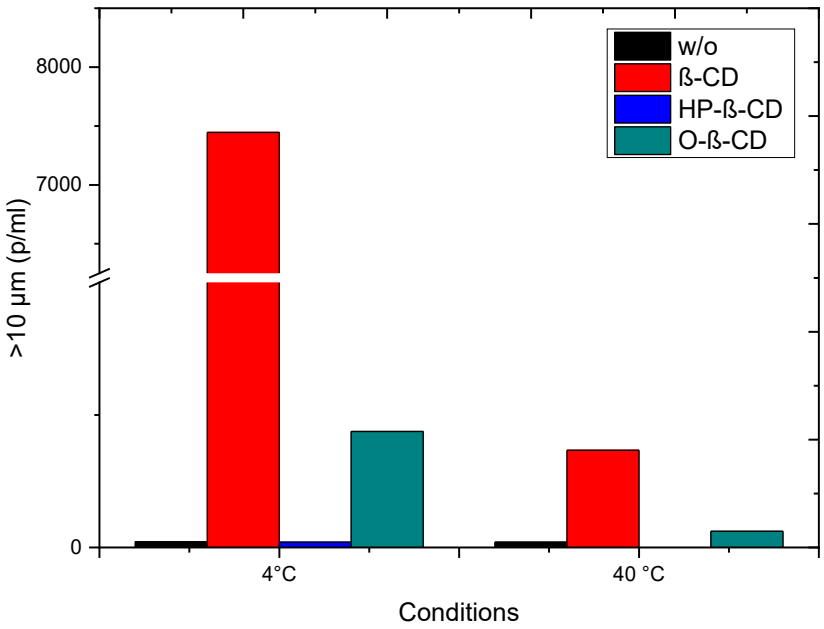


Figure 64 - Number of particles with diameter higher than 10 µm for PPB at pH 7.0 without any excipient (black), with β-CD (red), with HP- β-CD (blue), with maltoheptaose (green;) stored at 4°C (first group) and 40°C (second group)

The FlowCam has also been used to evaluate the particle number in solution of β-CD in ABS and PPB with different concentrations, to decide whether the excipient can be studied in lower concentrations in subsequent studies. According to this analysis, in ABS at pH 4.5 the maximum possible concentration would be 5 mM, but it is not clear whether such low a concentration could really protect the protein, effectively realizing preferential inclusion. In potassium phosphate buffer at pH 7.0, the particles number was dramatically high when the excipient concentration is 15 mM, otherwise the amount was not that relevant. These results are consistent whit that has been previously observed and discussed.

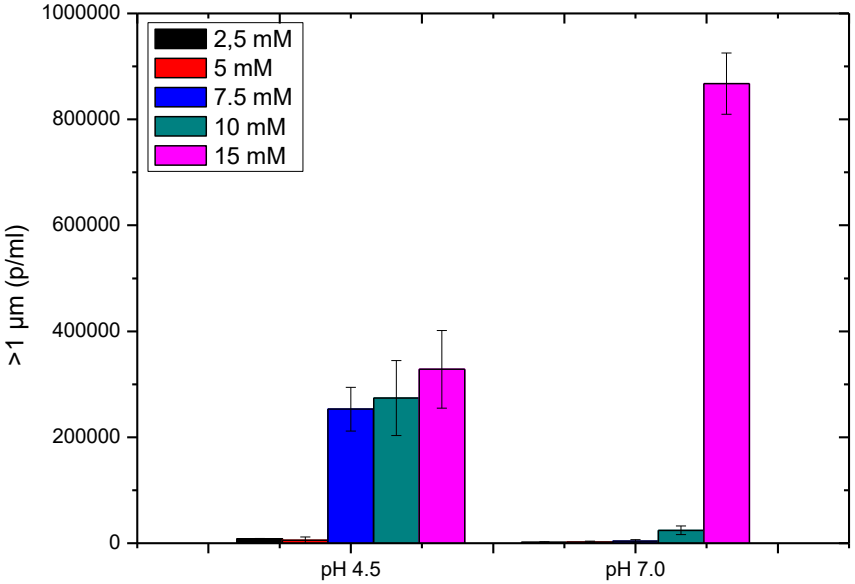


Figure 65 - Number of particles with diameter higher than 1 µm for ABS at pH 4.5 with β-CD at different concentrations: 2,5 mM (black), 5 mM (red), 7.5 mM (blue), 10 mM (green), 15 mM (pink)

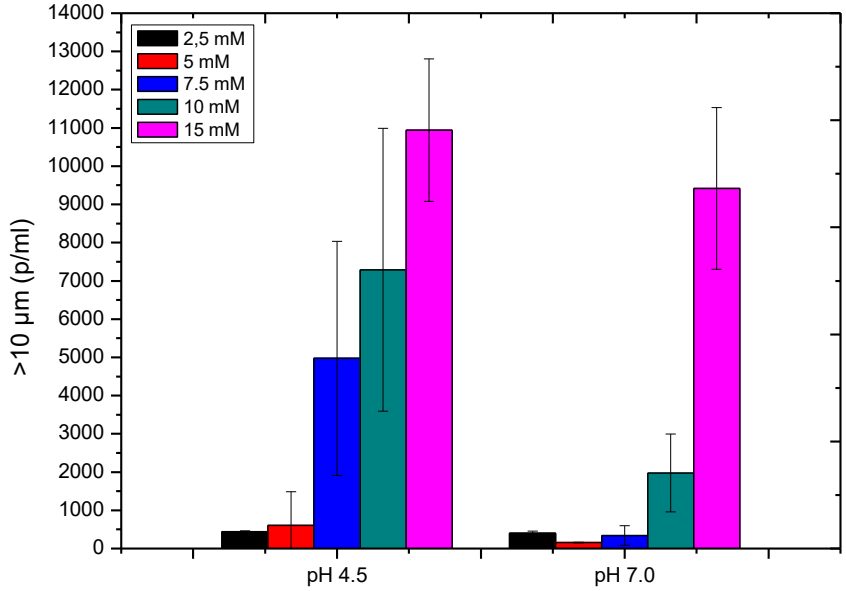


Figure 66 - Number of particles with diameter higher than 10 µm for ABS at pH 4.5 with β-CD at different concentrations: 2,5 mM (black), 5 mM (red), 7.5 mM (blue), 10 mM (green), 15 mM (pink)

3.2. Turbidity

The turbidity has been measured to further investigate the contribution of the β-CD to the presence of particles in formulations and to support the results from flow imaging microscopy. It is presented in terms of absorbance of solutions at wavelength of 350 nm: the higher the absorbance, the higher the turbidity.
The trend of turbidity of ABS buffer with and without excipient is consistent to what has been found in the previous analysis, although the value of absorbance for 2.5 mM solution is the highest one. It is reasonable to think that it could be due to a scratch on the bottom of the plate. Thus, again the maximum concentration of β-CD is 5 mM, which turbidity is comparable with the buffer without any excipient.

In PPB the turbidity showed a β-CD concentration dependence and the values are significantly lower than at pH 4.5.

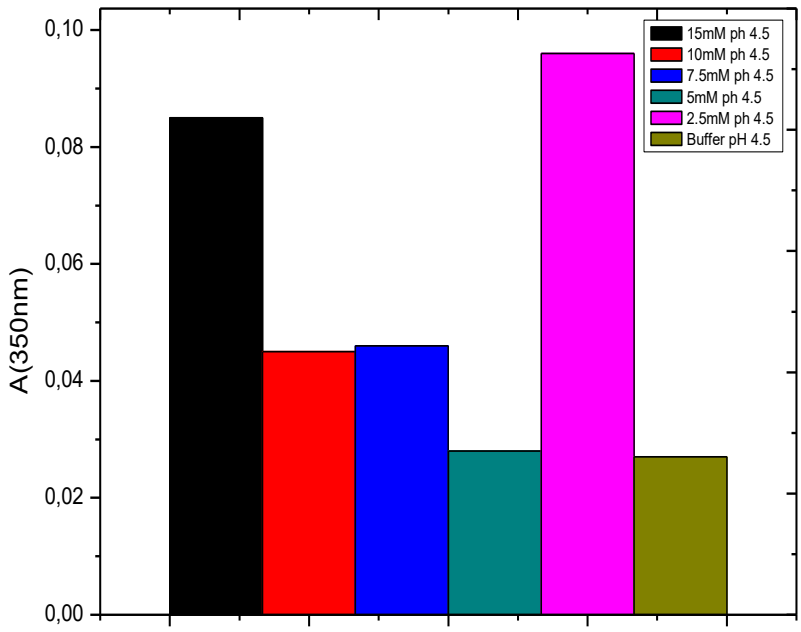


Figure 67 – Turbidity in terms of absorbance of ABS at pH 4.5 with β-CD at different concentrations: 15 mM (black), 10 mM (red), 7.5 mM (blue), 5 mM (green), 2.5 mM (pink), 0 mM (olive green)

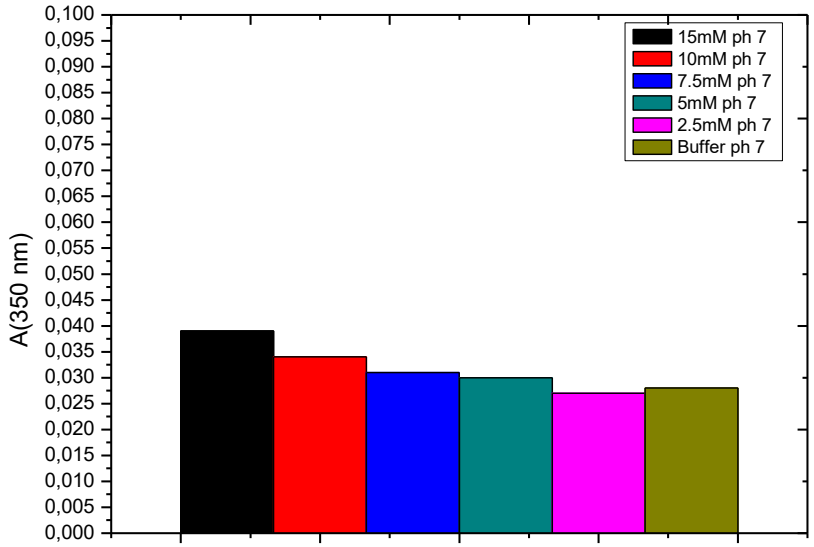


Figure 68 - Turbidity in terms of absorbance of PPB at pH 7.0 with β-CD at different concentrations: 2,5 mM (black), 5 mM (red), 7.5 mM (blue), 10 mM (green), 15 mM (pink)

Chapter 4

4. Conclusions and future perspectives

The aim of this project was the investigation of the use of cyclodextrins as excipients in protein formulations. The basis of this work lies on the molecular dynamic simulations performed at Politecnico di Torino. The experimental data set has investigated the direct binding of the excipient to the protein and many characteristics linked to the protein stability. In fact, the conformational and thermodynamic stability of proteins is linked to the probability of protein denaturation or aggregation to occur.

4.1. GCSF

The binding of the excipients to GCSF has been investigated through BLI and MST. The direct binding was mostly expected for β-CD, which, according to MD simulations, should be included by the protein. However, the intensity signal was weak. The clearest binding signal has been observed for HP-β-CD and it suggests a concentration dependence either in BLI assays, either in MST experiments; at pH 7.5 no binding signal was appreciable. This could be due to the enhanced propensity of the protein to aggregate in correspondence of this pH value; thus, the protein which lose its native structure is less binding-prone. These results stress the importance of pH in protein formulations because it strongly influences protein behaviour and protein-excipient interactions.

The protein stability has been investigated after preparation of formulations and after accelerated stability tests.

Closed and open form of β-CD do not affect the protein structure right after formulation.
The activity of the protein formulated with those excipients is preserved when the samples are shaken, freeze-thawed or stored at 4°C; while the activity is less preserved when the samples are stored at 40°C or lyophilized. Furthermore, lyophilized cake has collapsed when the protein has been formulated with β-CD. Therefore, it is possible to conclude that β-CD is not a good lyoprotectant. This hypothesis is also supported by the results concerning aggregation: the aggregates content is high when the protein formulated with β-CD is lyophilized and stored afterwards.
It has also been highlighted that the presence of huge number of particles in β-CD formulations is attributable to the solubility of the excipient it-self. The particles amount measured in the buffer is lower when the temperature increases (the solubility is enhanced), when the concentration of excipient decreases or when the pH is higher. The turbidity has shown the same trend.
This conclusion goes in the opposite direction from the results of the MD simulations, which showed less propensity to aggregation either when proteins interact with β-CD

(open and closed form) or when the excipient is self-interacting with respect to the hydroxypropyl form.
The hydroxypropyl form of β-CD does not affect the native structure of the protein when the formulation is not subjected to any stress. The excipient seems to be the most effective against chemical and thermal denaturation: T_m and T_{agg} are slightly higher when GCSF is formulated with HP-β-CD and the concentration of denaturing agent (Urea), necessary to unfold the structure, is higher as well.
HP-β-CD is known as potential lyoprotectant and the investigation of this aspect is one of the aims of this project. The excipient protects the formulations from pH shift during lyophilization. The formation of aggregates in freeze-dried samples is inhibited when the protein is formulated with the excipient, except after storage at 40°C, and the activity of the protein is preserved. However, it has been possible to notice the possible formation of oxidized species in samples containing HP-β-CD.

HP-β-CD seems to be the most promising among the excipients tested. It has shown slight propensity to enhance thermal and chemical stability of GCSF; thus, it may be interesting to further explore these aspects. For example, the chemical stability could be investigated at higher excipient concentration or with different denaturing agent (e.g.: guanidinium chloride), while it is possible to know more about the thermal stability testing different excipient concentration as well and comparing the results about T_m with that is obtained with different techniques, like differential scanning calorimetry (DSC) which principle and functioning are widely explained in literature. Furthermore, the use of HP-β-CD as cryoprotectant and lyoprotectant deserves to be further investigated, because lyophilization is one of the most utilized process to improve the storage stability of biopharmaceuticals, even though it does not seem to be the best option for GCSF, because it affects the protein activity.

The study highlighted the limits of DLS in this specific case of study: it does not give a clear size distribution of the protein, because of the diffusion of cyclodextrins. This issue was found in k_D determination, which is crucial in the evaluation of the propensity of the protein to either bind or aggregate.

4.2. LDH

In this study it has not been possible to investigate the direct binding of excipients to the protein. Thus, in order to understand whether cyclodextrins and maltoheptaose can protect the protein from denaturation that may occur during preparation, storage and transport of biopharmaceuticals based on LDH, thermodynamic and conformational stability of the protein has been investigated with different methods.

Tertiary structure of the protein is slightly modified by excipient and it is not appreciable significant difference among them.

None of the excipient enhanced thermal stability of the protein, while all of them prevent cake collapse during lyophilization.

Aggregation occurs when the protein is subjected to multiple freeze-thawing cycles or when the samples are stored at 40°C, even when they were previously lyophilized. The aggregation in liquid samples at 40°C was detected not only with proper techniques

but also as visible thin film, laying on the top of the liquid formulations. Thus, the excipients tested in this study did not show the ability to protect the protein formulated in PPB from denaturation induced by long term storage at high temperatures.

The activity of the protein when the initial concentration was 0.5 mg/mL was not affected by different stresses applied on the samples, was rather enhanced when the formulations had been subjected to low temperatures (freeze-thawing and freeze-drying). When the initial concentration of the protein was 100 times lower it was possible to appreciate similar trend, although less pronounced. Indeed, it was possible to notice that shaking causes important activity loss, freeze-thawing slightly reduces the activity, while it is completely preserved after lyophilization. This behaviour is attributable to the protein that, when high-concentrated, someway protect itself and it is alto perhaps related to reversible conformational changes, because when the activity of FT has been measured after 3 days of storage the values shown were lower.

Preventing denaturation at air-water interface seems to be crucial for this protein. From this point of view HP-β-CD is remarkable: only when the protein was formulated with this excipient it did not show any activity loss. HP-β-CD also prevented the little activity loss caused by freeze-thawing. The excipient protects the protein also from denaturation at ice-water interface.

Concerning HP-β-CD, when LDH has been formulated with this excipient the activity was always higher than the values shown by different formulations. It is possible to attribute this behaviour to a conformational change of the protein into a more active form, induced by the excipient.

For sure, the ability of HP-β-CD to act as surfactant and as lyoprotectant deserves to be further investigated. It may also be important to study in deep the phenomenon of activity increase after freeze-thawing cycles or freeze-drying, starting from different initial concentrations and trying to isolate the mechanism which causes the behaviour.

HP-β-CD is a promising excipient and it would be interesting to test is efficiency with other proteins.

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Abbreviations List

α-CD
β-CD
γ-CD
ΔG Change in Gibbs free energy
Θ Ellipticity
HP- β-CD
O- β-CD
RM- β-CD
SBE- β-CD
ABS Acetate Buffer Solution (Sodium Acetate Buffer)
Asn Asparagine
Asp Aspartic acid
BLI Biolayer Interferometry
CD Circular Dichroism
CDs Cyclodextrins
CMC Critical Micelle Concentration
Cys Cysteine
DLS Differential Scanning Fluorimetry
FT Freeze-Thawing
GCSF Granulocyte Colony Stimulating Factor
His Histidine
LDH Lactate Dehydrogenase
LYO Lyophilization
MST Microscale
NAD Nicotinamide Adenine Dinucleotide
NADH Reduced form of NAD
NaCl Sodium Chloride
NanoDSF Nano Differential Scanning Fluorimetry
MD Molecular Dynamics
PBS Phosphate Buffer Solution (Potassium Phosphate Buffer)
PD Polidispersity
PEG Polyethylene Glycol
PEI Polyethyleneimine
PVP Polyvinylpyrrolidone
RP-HPLC Reverse Phase High-Performance Liquid Chromatography
Tagg Aggregation Temperature
Tm Melting Temperature
Tg Glass transition Temperature
Trp Tryptophane
Tyr Tyrosine
UV Ultraviolet

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