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Glycosylation improves α-chymotrypsin stability upon encapsulation in poly(lactic-co-glycolic)acid microspheres

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ABSTRACT

Enhancing protein stability upon encapsulation and release from polymers is a key issue in sustained release applications. In addition, optimum drug dispersion in the polymer particles is critical for achieving release profiles with low unwanted initial "burst" release. Herein, we address both issues by formulating the model enzyme α-chymotrypsin (α-CT) as nanoparticles to improve drug dispersion and by covalently modifying it with glycans to afford improved stability during encapsulation in poly(lactic-co-glycolic) acid (PLGA) microspheres. α-CT was chemically modified with activated lactose (500 Da) to achieve molar ratios of 4.5 and 7.1 lactose-to-protein. The bioconjugates were co-lyophilized with methyl-β-cyclodextrin followed by suspension in ethyl acetate to afford nanoparticles. Nanoparticle formation did not significantly impact protein stability; less than 5% of the protein was aggregated and the residual activity remained above 90% for all formulations. Using a solid-in-oil-in-water (s/o/w) methodology developed in our laboratory for nanoparticles, we obtained a maximum encapsulation efficiency of 61%. Glycosylation completely prevented otherwise substantial protein aggregation and activity loss during encapsulation of the non-modified enzyme. Moreover, *in vitro* protein release was improved for glycosylated formulations. These results highlight the potential of chemical glycosylation to improve the stability of pharmaceutical proteins in sustained release applications.

1. Introduction

The sustained release of pharmaceutical proteins from poly(lactic-co-glycolic)acid (PLGA) microspheres for prevention and treatment of diseases has received wide interest [1–5]. Still, the encapsulation of proteins in the necessarily quite hydrophobic polymer matrix has remained challenging because the polymer is mostly dissolved in an organic solvent. Proteins are chemically and physically fragile and are susceptible to mechanical, thermal, and chemical stresses encountered in the encapsulation process. In this work we focus on improving physical instability issues during encapsulation which are characterized by protein structural changes potentially leading to subsequent irreversible inactivation and aggregation.

The most commonly employed polymer in sustained release applications of proteins is the family of PLGA co-polymers [6]. Water-in-oil-in-water (w/o/w), solid-in-oil-in-water (s/o/w), and solid-in-oil-in-oil (s/o/o) encapsulation are the most commonly used methods to incorporate proteins into PLGA microspheres [7]. The s/o/w encapsulation methods are advantageous when working with proteins because they avoid the first w/o interface encountered in w/o/w encapsulation which is particularly detrimental to protein integrity [4] and do not involve the use of an excess of organic solvent as in the s/o/o methods [8]. Unfortunately, also s/o/w encapsulation procedures are not free of protein stability issues likely due to the increased structural dynamics (flexibility) of the protein upon rehydration in the oil-in-water emulsion step [9]. Furthermore, the release of proteins from PLGA devices also produces difficulties, such as, protein instability due to exposure to PLGA hydrolysis products, high initial "burst" release, and incomplete protein release [5,10]. Protein aggregates are frequently formed during encapsulation and release and this must be avoided because they can cause dangerous immune reactions [9,11]. It has to be pointed out, however, that no maximum aggregate levels have been defined by the US Food and Drug Administration (FDA) and they should, in general, be kept as low as possible.

Some research has been performed focusing on eradicating protein aggregation and inactivation during the harsh encapsulation procedures and during release caused by PLGA-degradation produced acidification [1,7,12,13]. However, protein inactivation, aggregation, and unfolding during encapsulation are still issues severely hampering the application of sustained protein release PLGA microparticles [9].

To tackle protein stability problems during encapsulation in PLGA microspheres we engaged in a dual approach. First, we employed protein powders formulated as nanoparticles in a s/o/w encapsulation procedure. Drug particle size is highly relevant in this context because it can influence the bioavailability, loading, release, and stability of the drug. In s/o/w/ encapsulation reduced protein particle size should afford improved drug dispersion in the PLGA microspheres

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and improved release [14–16]. Second, we performed chemical glycosylation to improve thermodynamic and colloidal stability of our model protein. Covalent chemical modification (which includes modification with poly(ethylene glycol), carbohydrates, and cross-linking) is a promising approach to enhance protein stability in industrial and pharmaceutical applications [17–20]. The chemical glycosylation as performed by our laboratory consists in the modification of one or more protein lysine residues with chemically activated glycans [17,21]. Solá and Griebenow [22] demonstrated that increasing the size and amount of chemically attached glycans did not alter the structure of α-chymotrypsin (α-CT) employed as model enzyme herein but that a substantial decrease in protein structural dynamics and increase in stability was induced by glycosylation. Similar findings have also been reported by us for subtilisin Carlsberg [21].

In this study, we encapsulated glycosylated α-CT powders formulated as nanoparticles in PLGA microspheres by a s/o/w method. Protein stability was assessed as a function of the amount of bound lactose.

2. Materials and methods

2.1. Materials

α-Chymotrypsin (EC 3.4.21.1, type II from bovine pancreas), poly(vinyl) alcohol (87%–89% hydrolyzed, M_W of 13,000–23,000), and methyl-β-cyclodextrin (MβCD) were purchased from Sigma-Aldrich (St. Louis, MO). Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide was from Bachem (King of Prussia, PA) and poly(D,L-lactic-co-glycolic)acid (PLGA) with a copolymer ratio of 50:50 and an average M_W of 10,000 was from Lakeshore Biomaterials (Resomer RG502H, lot 260187, not endcapped). All other chemicals were from various suppliers and the purity of analytical grade or better.

2.2. Synthesis of α-CT glycoconjugates

Covalent modification of α-CT with lactose was performed as described in detail by Solá and Griebenow [22]. In brief, to attach various amounts of lactose to the enzyme, different amounts of activated lactose were added to a α-CT solution (4.5 and 7.1 mol of reagent per mol of protein) in 0.1 M borate buffer, pH 9.0 and stirred at 4 °C for 2 h. The glycoconjugates obtained were lyophilized and stored at −20 °C until further use. The degree of protein modification was determined by colorimetric titration of unreacted amino groups with 2,4,6-trinitrobenzene sulfonic acid (TNBSA) [23].

2.3. Preparation of conjugate nanoparticles

Formulation of α-CT as nanoparticles was performed as described in detail by Montalvo et al. [24]. In brief, α-CT and the lactose conjugates were dissolved in deionized water at 40 mg/mL protein concentration and methyl-β-cyclodextrin co-dissolved to achieve a 1:4 mass ratio of protein-to-cyclodextrin. These samples were lyophilized for 48 h and stored at −20 °C [23].

Protein nanoparticles were formed by suspending the lyophilized powders in 40 mL of ethyl acetate [24]. This suspension was sonicated for 30 s in an ultrasonic cleaning bath and the nanoparticles collected by centrifugation for 10 min at 7000 rpm and 4 °C in a Hermle Z 323 K with a Hermle Rotor # 220.80V02 from Labnet Int. (Woodbridge, NJ).

2.4. PLGA microsphere preparation

Microsphere preparation by a s/o/w encapsulation procedure followed the protocol developed by Griebenow and co-workers [25]. In brief, 40 mg of lyophilized α-CT powder or nanoparticles were suspended in 2 mL of ethyl acetate containing 360 mg of PLGA by homogenization with a VirTis Tempest using a 10-mm shaft (40,000 rpm, 30 s). This suspension was poured into 50 mL of PVA (10% w/v in distilled water) and the solid-in-oil-in-water emulsion was formed by homogenization (40,000 rpm, 2 min). Microspheres formed under stirring for 3 h. They were collected by filtration through a 0.45 μm pore size cellulose acetate filter, washed with 100 mL of distilled water, and dried for 24 h under a vacuum of <60 μm of Hg.

2.5. Determination of protein loading and encapsulation efficiency

The encapsulation efficiency was determined as described by us [8]. In brief, 20 mg of PLGA microspheres were dissolved in 2 mL of ethyl acetate and stirred for 2 h, followed by centrifugation at 9000 rpm for 10 min. The supernatant was discarded and the pellet vacuum dried for 30 min. The mostly of protein consisting pellet was dissolved in 2 mL of phosphate buffer. To separate the soluble and insoluble protein fractions, the samples were subjected to centrifugation at 9000 rpm for 10 min; the soluble fraction was removed and 1 mL of 6 M urea was added to the buffer insoluble-fraction to completely dissolve the protein aggregates. The protein concentration was determined by measuring the UV absorbance at 280 nm and by BCA assay at 562 nm. The encapsulation efficiency of protein in the microspheres was calculated from the actual loading with respect to the theoretical loading of protein (%w/w) in the microspheres. The experiments were performed in triplicate and the results averaged and the standard deviations calculated.

2.6. Determination of enzyme activity

Activity of α-CT was determined using succinyl-Ala-Ala-Pro-Phe-p-nitroanilide as the substrate. The reaction was carried out in 1 mL of 0.1 M Tris–HCl buffer containing 0.6 mg enzyme (protein), 0.35 mM substrate, and 0.01 M $CaCl_2$ at pH 7.8. To determine the activity of α-CT after encapsulation ethyl acetate was used to dissolve PLGA because it does not cause enzyme inactivation in the process [13]. The experiments were performed in triplicate and the results averaged. Error bars in the figures are the calculated standard deviations.

2.7. Scanning Electron Microscopy (SEM)

SEM of α-CT nanoparticles, PLGA microspheres, and lyophilized protein powder was performed using a JEOL 5800LV scanning electron microscope at a voltage of 20 kV. The samples were coated with gold (200–500 Å thickness). For each sample, the diameter of 100 microspheres was determined from images, averaged, and the standard deviation calculated.

2.8. Dynamic light scattering

Dynamic light scattering was performed using a DynaPro Titan with Ambient MicroSampler from Wyatt Technology Corporation (Santa Barbara, CA). Protein suspensions in ethyl acetate were added to the cell and measured at 100% power intensity. The data analysis was done using the Dynamic 6.7.6 software.

2.9. In vitro release studies

Microspheres (30 mg) were incubated in 1 mL of 10 mM phosphate buffer at pH 5.0 containing 0.05% sodium azide to prevent microbial growth and incubated at 37 °C [13]. At predetermined times the samples were subjected to centrifugation (5000 rpm for 5 min) to pellet the insoluble components. The supernatant was removed, the concentration of released protein determined, and the buffer replaced. Protein concentration was determined from the UV absorption at 280 nm and the small contribution due to eroding microspheres subtracted. For this purpose blank microspheres were subjected to *in vitro* release conditions [8,9].

2.10. FTIR spectroscopy

FTIR studies were conducted using a Nicolet NEXUS 470 optical bench as described in detail by us [26–29]. When necessary, the respective backgrounds were subtracted from the spectra acquired. Spectra were analyzed in the amide I spectra region as described by us including band assignment in Gaussian curve-fitting analysis [26–29].

2.11. Statistical methods

Statistical analysis was performed using Minitab 14 (Minitab Inc., State College, PA) software. The Dunn's test was applied to means of individual data statistical significance was accepted at the $p \leq 0.05$ level. The results presented in this work are the averages of at least three measurements and standard deviations were calculated for those data.

3. Results and discussion

3.1. Effect of glycosylation on α-chymotrypsin stability during s/o/w encapsulation

During s/o/w encapsulation proteins are exposed to an organic solvent/water interface which can cause protein structural perturbations and aggregation [2,9,29]. Even though protein powders are initially suspended in an organic solvent, during the o/w step of the procedure partial rehydration of protein particles will occur. This will increase protein conformational mobility and expose it to the denaturing stress imposed by the organic solvent [11,30,31].

In order to overcome these problems, herein we explore the glycosylation of the model enzyme α-CT to increase its stability during the encapsulation procedure. It has been previously demonstrated by us that the covalent chemical modification of α-CT and subtilisin Carlsberg with glycans is one of the most promising techniques to increase their stability since glycan–protein interactions lead to shielding of the protein surface from water thus restricting conformational motions [17,19–21]. Moreover, we also aimed at developing a drug delivery system with a relatively low burst release and improve the encapsulation efficiency. Our approach to reach this particular goal was to formulate the glycoconjugates as nanoparticles.

α-CT was chosen as the model protein since it has been employed previously by us to study the effect of glycosylation on enzyme stability including in the solid phase [17,18,21,22] and has been formulated as solid nanoparticles by us [24]. In addition, α-CT is an excellent sensor for encapsulation-induced aggregation and inactivation and has been employed by us frequently as model enzyme in s/o/w encapsulation procedures [13,29].

Lactose was covalently attached to α-CT using synthesis conditions adjusted to achieve an average number of lactose molecules bound to the protein of 4 (Lac_4-α-CT) and 7 (Lac_7-α-CT) since maximum thermodynamic and colloidal stability in solution have been reported for these constructs [18,22].

3.2. Protein nanoparticle preparation and characterization

To test whether we could form nanoparticles using the neoglycoconjugates, we co-dissolved α-CT and the α-CT glycoconjugates with methyl-β-cyclodextrin at a 1:4 mass ratio followed by lyophilization and suspension of the dry powders in ethyl acetate. The particles obtained were subjected to centrifugation and collected as described [24]. SEM images of α-CT lyophilized without MβCD show that the powder particles had an irregular shape and the particle size was in the micrometer range (Fig. 1A). In contrast, co-lyophilization with MβCD followed by suspension in ethyl acetate caused a drastic reduction in particle size for all formulations. α-CT nanospheres had a diameter of 115 ± 5 nm (Fig. 1B), Lac_4-α-CT nanospheres one of 248 ± 11 nm (Fig. 1C), and Lac_7-α-CT nanospheres one of 261 ± 4 nm (Fig. 1D) as determined by dynamic light scattering (Table 1). It was noticeable that the diameter of the particles approximately doubled as a consequence of the glycosylation. Nanoparticle formation did not compromise protein stability. The formation of buffer-insoluble protein aggregates was ≤5% for all the samples regardless of the modification. Furthermore, the residual activity of the samples did not change with exception of Lac_7-α-CT for which a 10% drop occurred (Table 1). All samples were subsequently employed to test the stability consequences of their encapsulation in PLGA microspheres.

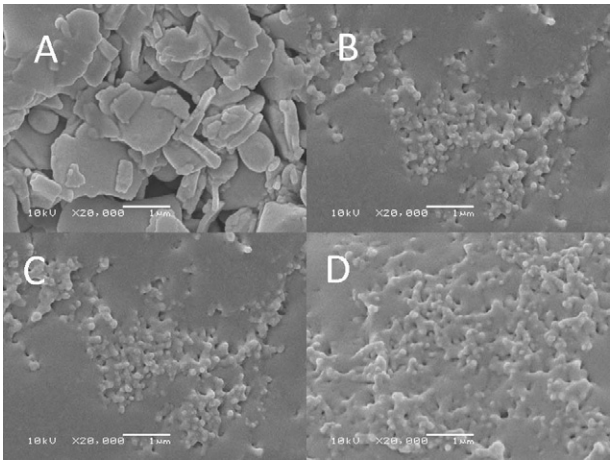

Fig. 1. SEM micrographs of lyophilized α-CT (A) and of nanoparticles formed using (B) α-CT, (C) Lac_4-α-CT, and (D) Lac_7-α-CT.

Table 1
Aggregate formation, residual activity, and particle size of non-modified and glycosylated α-chymotrypsin nanoparticles.

Sample	Noncovalent aggregates (%)	Residual activity (%)	Nanoparticle diameter (nm)
α-CT	2 ± 1	100 ± 2	115 ± 5
Lac_4-α-CT	5 ± 0	100 ± 2	248 ± 11
Lac_7-α-CT	3 ± 2	90 ± 2	261 ± 4

Table 2
Properties of PLGA microspheres prepared using different α-chymotrypsin formulations.

Sample	Encapsulation efficiency (%)[a]	Noncovalent aggregates (%)[b]	Residual activity (%)[c]	Microsphere diameter (μm)[d]
α-CT	30 ± 1	24 ± 2	53 ± 5	10–61
Lac_4-α-CT	61 ± 1	2 ± 1	100 ± 1	3–130
Lac_7-α-CT	23 ± 2	8 ± 2	84 ± 2	2–55

[a] Encapsulation efficiency is the percentage of encapsulated protein compared to the theoretical loading.
[b] Aggregated α-CT is the percentage with respect to the total amount of encapsulated protein.
[c] The activity (%) is the residual activity of α-CT released from PLGA microspheres.
[d] The microsphere diameter was obtained by analyzing SEM images.

3.3. Encapsulation of protein nanoparticles into PLGA microspheres.

Microspheres were prepared by a s/o/w technique using α-CT nanoparticles (Table 2). The encapsulation efficiency was between 23 and 61% allowing us to perform subsequent stability and release studies.

Protein stability during encapsulation in the PLGA microspheres was markedly improved by glycosylation. The amount of noncovalent buffer-insoluble aggregates formed during encapsulation

was 24% for the non-modified α-CT formulated as nanoparticles (Table 2) which is comparable to 18% reported for lyophilized α-CT [2,12]. A substantial reduction was noted for the encapsulated glycoconjugates: only 2% aggregates were found for encapsulated Lac_4-α-CT bound and 8% for Lac_7-α-CT. Reduction in aggregation has been attributed to the role of the glycans as spacer molecules preventing interactions of unfolded proteins [19,20].

Next, the effect of the nature of the glycosylation and nanoparticle formation on the morphology of α-CT-loaded PLGA microspheres was investigated. Encapsulation of all formulations produced microspheres with a spherical shape and smooth surface (Fig. 2A–C). The most significant difference between the different formulations was the size of the microspheres (Table 2). Microspheres with widely varying sizes were observed for all formulations (Table 2). An increasing amount of glycosylation of the enzyme (Lac_7-α-CT) caused a significant reduction in the size of the microspheres (Fig. 2C) which could be a reason for the low encapsulation efficiency observed for this preparation.

3.4. Activity of α-CT after encapsulation in PLGA microspheres

The residual α-CT activity was determined for the different formulations after encapsulation in PLGA microspheres. As a control, the residual activity for the different glycosylated formulations was determined prior to encapsulation to ascertain that the inactivation observed was caused by the encapsulation process and not by the initial lyophilization or nanoparticle formation step (Table 1). All glycosylated formulations exhibited higher α-CT activities than the non-glycosylated α-CT after encapsulation into PLGA microspheres (Table 2). The non-glycosylated nanoparticulate sample had a residual activity of 53 ± 5% after encapsulation which is comparable to 53 ± 8% found upon s/o/w encapsulation of lyophilized α-CT powder [12]. This demonstrates that the different mode of dehydration and formulation prior to encapsulation had no influence on enzyme stability while glycosylation caused a marked improvement of stability during encapsulation.

We assume that inactivation during encapsulation mainly stems from exposure of α-CT to the organic solvent in the presence of water. During encapsulation, specifically during formation of the o/w emulsion water enters the organic solvent phase and will hydrate the protein [29]. Such hydration results in increased protein structural mobility thus making it more amenable to irreversible unfolding and thus inactivation [2,19,20]. One can hypothesize that decreased conformational mobility as the result of glycosylation should counter such events [17,21,22]. Glycosylation indeed had a significant effect on preserving enzyme activity upon encapsulation. Remarkably, for Lac_4-α-CT we found complete retention of the activity upon encapsulation and for Lac_7-α-CT residual activity was >50% higher (84 ± 2%) than for the non-glycosylated protein. In summary, our data show that α-CT glycosylation leads to a remarkable increase of its stability upon s/o/w encapsulation in PLGA microspheres. These findings will have to be verified with another protein to establish whether this is a general phenomenon as we assume or protein specific.

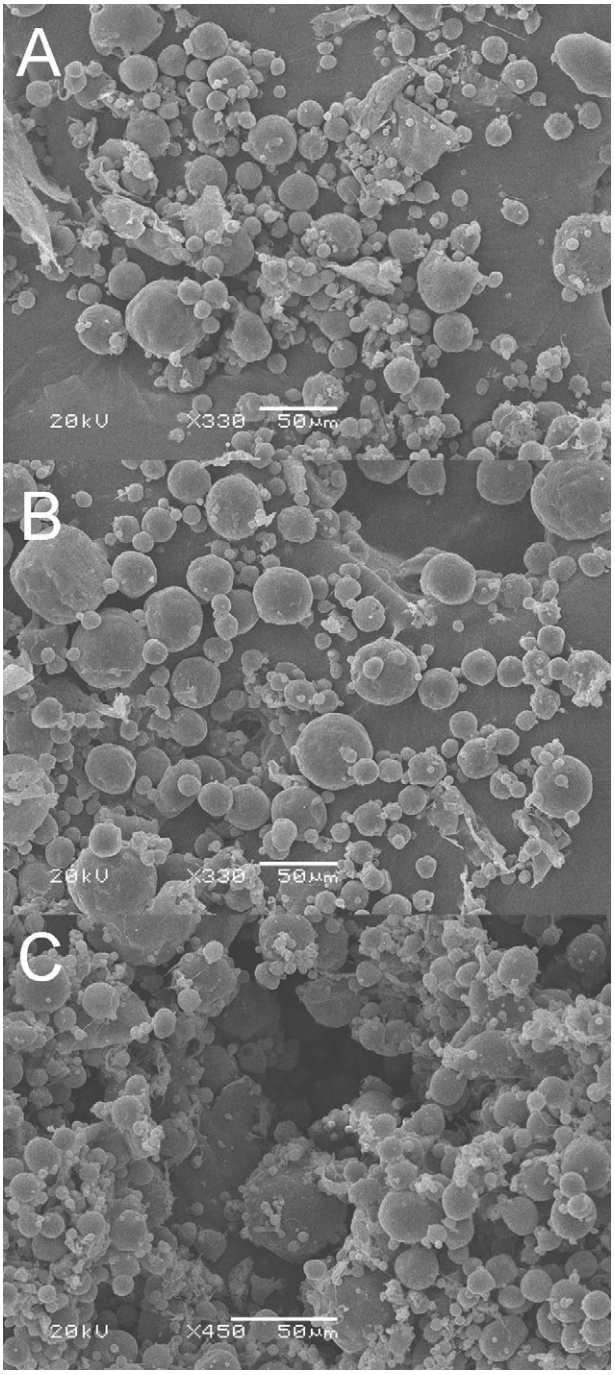

Fig. 2. SEM micrographs of PLGA microspheres with encapsulated (A) α-CT, (B) Lac_4-α-CT, and (C) Lac_7-α-CT.

3.5. Secondary structure of α-CT during the S/O/W encapsulation process

To investigate the potential mechanism of structural stabilization by glycosylation observed during encapsulation, we investigated the secondary structure of α-CT and the glycoconjugates encapsulated in PLGA microspheres by Fourier-transform infrared (FTIR) spectroscopy. In principal, glycosylation could afford structural preservation upon lyophilization and encapsulation (similar to lyoprotectants) [19] or simply work as molecular spacer keeping unfolded molecules apart and thus prevent aggregation and inactivation [18,22]. Two similar states of the samples were compared; nanoparticles before encapsulation and after the complete encapsulation process. The structurally sensitive amide I band (1600–1700 cm^{-1}) was utilized and subjected to a Gaussian curve-fitting procedure to extract the secondary structure composition [28].

α-CT secondary structure in aqueous solution is dominated by β-sheet structure (Table 3). Lyophilization leads to an apparent increase in the α-helix content and insignificant changes in the β-sheet content [32,33]. In contrast, a significant loss in β-sheet structure was found upon formulation as nanoparticles while encapsulation in PLGA

Table 3
Secondary structure composition of α-chymotrypsin under different conditions.

Sample/condition	α-helix[a] (%)	β-sheet (%)
α-CT		
Aqueous solution	14 ± 2	40 ± 2
Lyophilized powder	17 ± 2	41 ± 5
Nanoparticle	17 ± 2	29 ± 2
Nanoparticle in PLGA microspheres	17 ± 2	33 ± 2
Lac_4-α-CT		
Nanoparticle in PLGA microspheres	14 ± 2	33 ± 3
Lac_7-α-CT		
Nanoparticle in PLGA microspheres	17 ± 2	60 ± 2

[a] Unordered and α-helix secondary structure amide I IR bands overlap strongly in α-CT which causes an apparent increase in the α-helix content upon lyophilization.

microspheres did not cause major additional structural changes. Similar results were reported by Montalvo et al. [24] for α-CT, suggesting that this loss in β-sheet structure is mainly caused by the nanoparticle formulation and not by the encapsulation step.

Attachment of four lactose molecules to the enzyme caused no spectral and thus structural alterations compared to the non-modified protein, while attachment of seven lactose molecules caused a substantial increase in the β-sheet content. These results clearly demonstrate that the reduction in aggregation and inactivation upon encapsulation (Table 2) for the glycoconjugates is not being caused by a lyoprotectant effect of the attached lactose molecules. Thus, it is likely that the beneficial stability increase is being caused by the spacer effect afforded by the lactose molecules keeping molecules apart. For Lac_7-α-CT it seems that the stability increase afforded by this effect is partially ameliorated by structural changes upon dehydration. Stability and FTIR data agree in that Lac_4-α-CT is the most stable formulation.

3.6. *In vitro release studies*

Proteins are released from PLGA microspheres first by polymer swelling and diffusion and then also by polymer erosion. One of the main challenges of the development of a sustained release system based on polymer microspheres is minimizing the initial "burst" release [9,34,35]. One reason for burst release after s/o/w encapsulation is that protein particles are close to the microsphere surface and solvent enters the polymer through small pores of the polymer matrix dissolving solvent accessible protein. For example, it has been demonstrated that microspheres showing high burst release have a more porous surface than those with low burst release [36].

In vitro release studies were conducted investigating the magnitude of burst release from PLGA microspheres containing encapsulated nanoparticles compared to conventional lyophilized powder. The cumulative release profiles of microspheres loaded with α-CT nanoparticles and glycosylated α-CT nanoparticles are shown in Fig. 3. Microspheres loaded with α-CT nanoparticles showed a burst release of 30% during the first 24 h which is lower than the 50% burst release reported by us for the lyophilized powder using the same encapsulation conditions [12]. Lyophilized protein powders typically produce much larger protein particles in the first s/o encapsulation step of micrometer dimensions. This leads to a substantial burst release when microspheres are produced by a s/o/w methodology and has hampered practical development thus far [9,37]. Encapsulated nanoparticles of Lac_4-α-CT and Lac_7-α-CT showed an even further reduced burst release of 20% and 17%, respectively (Table 2). Our data demonstrate that the burst release was reduced by employing nanoparticulate protein powders.

A triphasic *in vitro* release of α-CT from PLGA microspheres was observed for all formulations; the initial burst release was followed by a lag phase and a period of sustained release (Fig. 3). The release profiles were similar for all formulations employing nanoparticles with the exception that the release was more complete for the glycosylated formulations.

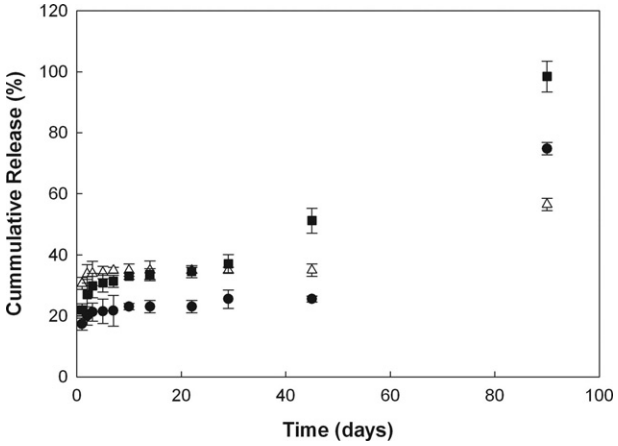

Fig. 3. Cumulative *in vitro* release of (Δ) α-CT, (■) Lac_4-α-CT, and (•) Lac_7-α-CT from PLGA microspheres.

Table 4
Residual activity of α-CT-lactose conjugate after various times of *in vitro* release from PLGA microspheres.

Time (h)	α-CT	Lac_4-α-CT	Lac_7-α-CT
24	45 ± 2	46 ± 2	40 ± 2
48	31 ± 1	38 ± 2	43 ± 4
72	n.a.	17 ± 1	24 ± 1
96	n.a.	18 ± 1	n.a.

n.a.: no measurable activity.

The relative activity of α-CT released from microspheres was followed for 1 week. Table 4 shows that the non-glycosylated α-CT maintained activity only for the first 48 h. In contrast, Lac_4-α-CT retained 18% of its activity for 96 h and Lac_7-α-CT 24% of activity for 72 h. Glycosylation of α-CT afforded some but only incomplete protection of the activity upon *in vitro* release. α-CT is inactivated during prolonged incubation at 37 °C due to fragmentation and glycosylation does not protect against that [13].

4. Conclusions

In this work we investigated whether glycosylation of the model enzyme α-CT could be used to improve protein stability upon encapsulation into PLGA microspheres. α-CT was chemically modified with activated lactose to achieve molar ratios of 4.5 and 7.1 lactose-to-protein and formulated as spherical nanoparticles of about 250 nm diameter. Non-modified and glycosylated α-CT nanoparticles were subsequently encapsulated in PLGA microspheres using a s/o/w methodology. We found that glycosylation was able to completely prevent otherwise substantial protein aggregation and activity loss during encapsulation. These results highlight the potential of chemical glycosylation to improve the stability of pharmaceutical proteins in sustained release applications.

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