Characterization of Peptide Polymer Interactions in Poly(alkylcyanoacrylate) Nanoparticles: A Mass Spectrometric Approach

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Abstract

Drug/polymer interactions occur during *in situ* polymerization of poly(alkylcyanoacrylate) (PACA) formulations. We have used MALDI ionization coupled tandem time-of-flight (TOF) mass spectrometry as an accurate method to characterize covalent peptide/polymer interactions of PACA nanoparticles with the bioactives D-Lys^6-GnRH, insulin, [Asn^1-Val^5]-angiotensin II, and fragments of insulin-like growth factor 1 (IGF-1 (1-3)) and human adrenocorticotropic hormone (h-ACTH, (18-39)) at the molecular level. Covalent interactions forming peptide/PACA co-polymers were identified for D-Lys^6-GnRH, [Asn^1-Val^5]-angiotensin II and IGF-1 (1-3). D-Lys^6-GnRH and [Asn^1-Val^5]-angiotensin II were modified at their histidine side chain within the peptide, whilst IGF-1 (1-3) was modified at the C-terminal glutamic acid residue. The more complex protein insulin did not co-polymerize despite the presence of 2 histidine residues. This might be explained by the engagement of histidine residues in the folding and sterical arrangement of insulin under polymerization conditions. As expected, h-ACTH (18-39) that does not contain histidine residues did not co-polymerize. Lowering the pH did not prevent the co-polymerization of PACA with D-Lys^6-GnRH or IGF-1 (1-3). Conclusively, protein and peptide bioactives are potentially reactive towards PACA nanoparticles via various mechanisms with limited interference of pH. Histidines and C-terminal glutamic acid residues have been identified as potential sites of interaction. The likelihood of their engagement in co-polymerization, however, seems dependant on their sterical availability. The potential for co-polymerization should be

considered when designing a PACA delivery system for protein and peptide biopharmaceuticals.

Keywords (8): MALDI TOF/TOF, co-polymerization, PACA, insulin, IGF-1, GnRH, angiotensin, h-ACTH

1. INTRODUCTION

Poly(alkylcyanoacrylate) (PACA) nanoparticles appeal to formulation scientists for a number of reasons. Their potential to incorporate both hydrophobic and hydrophilic compounds by utilizing different preparation techniques renders them applicable to a wide range of drugs [1, 2]. The number of protein and peptide drugs is increasing as the sector of modern biopharmaceuticals is growing, demanding pharmaceutical formulations capable of ensuring their safety, stability and bioactivity [3]. High entrapment efficiencies (~85%) of peptide bioactives, such as insulin [4, 5] and calcitonin [6] have been achieved using the anionic interfacial polymerization technique to produce PACA nanoparticles. Using biodegradable and biocompatible w/o-microemulsions as polymerization templates is particularly beneficial for PACA formulations with peroral applications since microemulsions exhibit permeation enhancing properties [7]. Comprehensive reviews on their biomedical applications as controlled drug delivery systems are available elsewhere [1-3, 8].

The formation of polymeric nanoparticles by anionic interfacial polymerization is usually initiated by slowly adding monomer to the polymerization template (e.g. a w/o-microemulsion). The hydroxyl ions arising from the autoprotolysis of water are believed to be the main reaction initiators for the anionic reaction pathway [1, 9]. *In situ* polymerization requires all ingredients, including both the monomer and the drug, to be present during the polymerization step. Thus, the drug has the potential to interfere with the polymerization process. For some polymeric PACA systems produced by *in situ* polymerization, incomplete

release (less than 80%) of bioactive was suggested to be indicative of strong drug-polymer interactions causing certain drug fractions to remain unreleased [10, 11]. The release of a gonadotropin releasing hormone (GnRH) analogue from poly(ethylcyanoacrylate) (PECA) nanoparticles was as low as 10% when incubated in phosphate buffer pH 7.4 for several days [12]. Such release characteristics may impair the bioavailability and effectiveness of the drug, depending on therapeutic doses and action onset times required. However, covalent association of drug with polymer (co-polymerization) may also function as a means to produce sustained and controlled release delivery systems with slow release profiles. Previous research has involved the deliberate functionalization of bioactives in order to add a reactive entity capable of covalently interfering with the polymerization process, such as the addition of vinyl acetate to the bioactive GnRH [13] or the addition of an acryloyl-functional group to the antigenic epitope of the lymphocytic choriomeningitis virus glycoprotein (LCMV33-41) [9]. The resulting co-polymerized systems demonstrated improved stability of the bioactive in physiological fluids [13] and prolonged slow-release characteristics via bioerosion [14]. Therefore, such co-polymerized nanoparticulate delivery systems seem particularly suitable for sustained drug delivery, such as delivery of vaccines [15].

Methods employed to investigate the phenomenon of co-polymerization in PACA nanoparticles include (i) size exclusion chromatography (SEC), more specifically gel permeation chromatography (GPC) [14] and (ii) thin-layer chromatography (TLC) [11]. Chemical modification of polymer fractions were inferred due to (i) the occurrence of an earlier

eluting fraction (i.e. larger size) causing a shoulder peak in the GPC profile compared to a reference chromatogram obtained by a physical mixture of preformed polymer and drug [14] and (ii) the occurrence of additional bands in the thin-layer chromatogram compared to reference bands of empty polymers, drug alone or a physical mixture of drug and polymer [11]. These methods, however, have a number of limitations. Both chromatographic methods separate the analytes according to bulk characteristics, such as the hydrodynamic volume (SEC) or affinity of the analyte towards certain surfaces (e.g. silica-grafted surfaces as in TLC). The detection of signals based on the refractive index of eluents in SEC or UV-Vis visualization of bands in TLC are unselective and molecular species co-eluting at the same will contribute to the same signal/band in the chromatogram. Unambiguous identification of analytes is lacking in these methods. Furthermore, in order to interpret a chromatogram, reference chromatograms are required. Depending on the preparation of reference samples, physical mixtures of preformed PACA nanoparticles and drug may still retain the potential to form covalent drug-polymer bonds whilst in the liquid phase. In our recent studies, we have demonstrated the potential of a GnRH-analogue to co-polymerize with PECA nanoparticles when added 4 hours after the polymerization reaction was completed (*ex situ* addition) [12]. Interpretations of reference chromatograms with the assumption that no peptide-polymer interactions occur in a physical mixture can lead to misinterpretations.

We recently employed matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI TOF/TOF MS) in combination with collision-induced dissociation

(CID) to characterize covalent interactions of PECA nanoparticles with D-Lys^6-GnRH after *in situ* and *ex situ* polymerization [12]. MALDI coupled TOF/TOF MS proved to be particularly suitable for the analysis of peptide/PECA co-polymers in the presence of excess PECA polymer. In MALDI, a soft ionization method that enables the measurement of large intact biomolecules and polymers, samples are co-crystallized with excess matrix, a compound that is activated upon absorption of laser energy. A mixture of activated matrix (here a cinnamic acid derivative) and analyte is vaporized and transferred into the gaseous phase where a proton transfer between activated matrix and analyte occurs yielding mostly singly charged ions (z = 1). Prerequisite for MALDI ionization is the ability of an analyte to donate or accept protons from a given matrix. D-Lys^6-GnRH and its conjugated co-polymers showed a very efficient ionization, while ionization of PECA polymers was strongly suppressed. The covalent interaction of the histidine residue in D-Lys^6-GnRH with PECA was identified unambiguously by CID-based tandem TOF MS. In the first TOF analysis, accurate masses of unmodified peptide and molecular species shifted by a multiple of 125 mass units corresponding to the mass of PECA monomers were detected. The site of interaction was then analyzed by mass measurements of fragment ions after collision induced dissociation of selected co-polymers. Mass spectrometry of CID-based ion fragments reveals sequence information and information about sites of amino acid modifications.

In the present study we extend our previous work to gain a more detailed insight into the possible reaction mechanisms involved. Using MALDI TOF/TOF MS in combination with

CID we investigate a range of histidine-containing and non-histidine-containing bioactive peptides on their capability to covalently interfere in the polymerization process of different poly(alkylcyanoacrylates), namely ethyl- and butylcyanoacrylate. We further tested how the pH, hence ionization status of peptides at the time of polymerization, influences the co-polymerization with PECA nanoparticles.

2. EXPERIMENTAL

2.1. Reagents

The GnRH analogue, D-Lys^6-GnRH (p-Glu-His-Trp-Ser-Tyr-D-Lys^6-Leu-Arg-Pro-GlyNH_2) was purchased from PolyPeptide Laboratories (Torrance, CA, USA). The angiotensin II analogue, [Asn^1, Val^5]-angiotensin II (Asn^1-Arg-Val-Tyr-Val^5-His-Pro-Phe) was purchased from Sigma-Aldrich Inc. (St Louis, MO, USA). The commercially available Humulin® R (Eli Lilly Australia Pty. Limited, NSW, AU) contains 100 IU/ml recombinant human insulin, stabilized by m-cresol and glycerol at a pH of 7.4. Humulin® R solution was concentrated 2.5 times using a centrifugal vacuum concentrator (Savant SpeedVac Plus SC 210 A). IGF-1 (1-3) fragment (Gly-Pro-Glu) was purchased from Bachem AG (Bubendorf, CH). The peptide fragment (18-39) of human adrenocorticotropic hormone (h-ACTH) (Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe) was purchased from Sigma-Aldrich (St Louis, MO, USA). The monomers ethylcyanoacrylate

(ECA) (Sicomet 40) and butylcyanoacrylate (BCA) (Sicomet 6000) were kindly donated by Henkel Loctite (Hannover, Germany).

For preparation of microemulsions, the oil component ethyloleate GPR was sourced from BDH Laboratory Supplies (Poole, England) and the surfactants sorbitan monolaurate (Crill 1) and ethoxy 20 sorbitan mono-oleate (Crillet 4) were kindly provided by BTB Chemicals (Auckland, New Zealand). Methanol (HPLC grade), chloroform, acetonitrile (HPLC grade) and hydrochloric acid (fuming 37%, GR for analysis) were obtained from Merck KGaA (Darmstadt, Germany). Ethanol was supplied by AnchorEthanol (Auckland, New Zealand). Distilled, ultra-pure water was obtained from a Milli-Q water Millipore Purification System (USA) and was used at all times during sample preparation.

2.2. Preparation of PECA nanoparticles from a microemulsion template

For preparation of a microemulsion template, we followed the method of Watnasirichaikul *et al*. [4] mixing water, oil and a surfactant-blend together at a 1 : 3.6 : 5.4 weight ratio. Beforehand, the surfactants Crill 1 and Crillet 4 were mixed in a 4 : 6 weight ratio to form the surfactant-blend. The bioactive peptides (D-Lys^{6}-GnRH, insulin, IGF-1 (1-3), [Asn^{1}-Val^{5}]-angiotensin II and h-ACTH (18-39)) were each dissolved in the water phase prior to mixing with the other microemulsion ingredients. Important peptide characteristics and amounts of peptides used in this study are given in Table **1**. Distilled, ultrapure water had a pH of 6.3 and was adjusted for studying the influence of pH to 1.9 with hydrochloric acid.

Microemulsion formation occurred instantly upon stirring at 700 rpm at 4°C. 10 g of microemulsion was polymerized with 200 µl ECA monomer dissolved in 600 µl of chloroform. The monomer was added slowly to the microemulsion template under continuous stirring at 700 rpm at 4°C. After the addition of monomer, the mixture was left overnight to complete the polymerization process and to evaporate the chloroform. 0.5 g of formulation was mixed with 2.5 ml ultrapure water pH 2.5 (pH adjusted with hydrochloric acid). An aliquot of 600 µl was withdrawn and mixed with 600 µl methanol 80% (v/v, water pH 2.5) allowing for the separation of nanoparticles from the surrounding microemulsion by centrifugation at 20,800 g and 25°C for 20 min (Eppendorf Centrifuge 5415C). Nanoparticles were subsequently washed twice with in ethanol (abs.) to remove residual oil and surfactants. Isolated nanoparticles were characterized using MALDI TOF MS/MS the day after polymerization.

Table 1. Relevant formulation and peptide characteristics

2.3. MALDI TOF mass spectrometry

Freshly prepared and washed nanoparticles were dissolved in approximately 20-40 µl acetonitrile and 1.0 µl of sample solution was mixed with 9.0 µl of matrix (10 mg/ml alpha cyano-4-hydroxycinnamic acid dissolved in 60% (v/v) aqueous acetonitrile containing 0.1% (v/v) trifluoroacetic acid). An aliquot of 0.8 µl was spotted onto the MALDI-plate (Opti-TOF

384 well plate, Applied Biosystems, MA, USA) and air-dried. Samples were analyzed on a 4800 MALDI tandem Time-of-Flight Analyzer (Applied Biosystems, MA, USA). The default calibration for each operation mode was updated on six calibration spots. All MS spectra were acquired in positive-ion reflector mode with 1000 laser pulses per sample spot.

Co-polymers were identified by the presence of mass signals $[M + (P)_n + H]^+$, corresponding to parent peptide mass $[M + H]^+$ being shifted by a certain number of polymer increments $(P)_n$. To identify the amino acid involved in peptide/PECA or PBCA interaction co-polymer precursor ions were selected for collision induced dissociation tandem mass spectrometry (CID-MS/MS). CID-MS/MS spectra were acquired with 2000-4000 laser pulses per selected precursor using the 2 kV mode. Air was used as collision gas at a pressure of $1 \cdot 10^{-6}$ torr. CID-MS/MS spectra were interpreted manually as described previously [12] mainly considering b-, y- and a-type ions as well as relevant immonium ions according to the Biemann nomenclature [16].

Table 2. Summary of peptide co-polymerization with ethyl- and butylcyanoacrylate

3. RESULTS

3.1. Co-polymerization of different bioactive peptides with ethyl-and butylcyanoacrylate

Three of the five bioactive peptides investigated were found to co-polymerize with both ethyl-and butylcyanoacrylate (Table **2**). Co-polymerization was determined by the occurrence of co-polymer signals $[M + (P)_n + H]^+$ in the MS spectra for all three peptides (Fig. **1a-c**). MS/MS spectra were recorded for selected co-polymer precursor-ions in order to identify the site of modification within the peptide molecule (Table **2**). Relevant spectra of modified histidine- and non-histidine-containing peptides are discussed in more detail in the following sections.

3.1.1. Histidine-containing peptides with ethyl-and butylcyanoacrylate (D-Lys^6-GnRH, [Asn^1, Val^5]-angiotensin II and insulin)

The previously identified reactivity of D-Lys^6-GnRH to co-polymerize with ethylcyanoacrylate [12] was also demonstrated for butylcyanoacrylate (Fig. **1a)**. Unmodified D-Lys^6-GnRH gave a peak at m/z 1253.6 $[M + H]^+$, which indicated that the peptide within the PBCA nanoparticles existed, in part, in the free form. Relative to the mass of the free peptide $[M + H]^+$, co-polymers of D-Lys^6-GnRH with PBCA were shifted by a multiple of monomer subunits $(153)_n$ (m/z 1559.8 for 2, m/z 1712.9 for 3, m/z 1866.0 for 4, m/z 2019.1 for 5, m/z 2172.1 for 6, etc.). CID-MS/MS on selected precursor ions of co-polymers revealed the histidine residue in position 2 of D-Lys^6-GnRH as the site of modification (Table **2**).

Fig. (1)

[Asn^1-Val^5]-angiotensin II, another small histidine-containing peptide, also co-polymerized when polymerized *in situ* with PECA and PBCA nanoparticles. Mass spectra of [Asn^1-Val^5]-angiotensin II recorded for PECA and PBCA are shown in Fig. **1b**, and **1c** respectively. Similar to D-Lys^6-GnRH, unmodified [Asn^1-Val^5]-angiotensin II entrapped within the polymeric nanoparticles was identified at m/z 1031.5 $[M + H]^+$. Co-polymers were detected as a series of ions shifted either by increments of 125 mass units for ECA (Fig. **1b**) or 153 mass units for BCA (Fig. **1c**). Precursor ions (*) of the peptide/PECA co-polymer series at m/z 1406.7, 1531.7 and 1656.8 corresponding to $[M + (125)_{3\text{-}5} + H]^+$ were selected for CID-MS/MS (Fig. **2**).

Fig. (2)

CID-based fragmentation of co-polymers yielded dissociation of peptide (m/z 1031.5) from polymer as well as sequential loss of monomer subunits $[\text{Precursor} - (P)_n]^+$ (Fig. **2a-c**). For example, the fragment spectrum of precursor-ion m/z 1656.8 that represents [Asn^1-Val^5]-angiotensin II co-polymerized with 5 monomers $[1030.5 + (125)_5 + H]^+$ (Fig. **1b**) shows the loss of 1 (m/z 1531.7), 2 (m/z 1406.7), 3 (m/z 1281.6) and 5 (m/z 1031.5) monomer subunits (Fig. **2c**). CID-based fragmentation of the peptide backbone revealed information of the site of

polymer addition to the peptide. A clear signal of modified histidine immonium ions identified the histidine residue as the site of modification (Fig. **2a-c**). Amino acid-specific immonium ions are low mass fragment ions that are generated by multiple cleavage events and contain only a single amino acid side chain. Such immonium ions can be used as diagnostic ions to confirm the presence of certain amino acids. Histidines usually generate strong signals of their specific immonium ion at m/z 110. In all CID spectra of [Asn^1-Val^5]-angiotensin II/PECA co-polymers, the histidine immonium ion was shifted by the mass corresponding to the number of PECA monomers associated with the peptide. For example, histidine immonium ions in CID spectra of precursors (*) m/z 1406.7 $[1030.5 + (125)_3 + H]^+$, 1531.7 $[1030.5 + (125)_4 + H]^+$ and 1656.8 $[1030.5 + (125)_5 + H]^+$ appeared at m/z 485.2 $[110 + (125)_3]^+$(Fig. **2a**), m/z 610.3 $[110 + (125)_4]^+$(Fig. **2b**), and m/z 735.3 $[110 + (125)_5]^+$ (Fig. **2c**). No other amino acid was found to be modified. This confirms the histidine residue in position 6 of the peptide molecule is the only site of modification. The same trend was seen in the CID-MS/MS spectra of the [Asn^1-Val^5]-angiotensin II/PBCA co-polymers (data not shown).

In contrast to the small histidine-containing peptides tested here, no co-polymerization was observed when the more complex, histidine-containing protein insulin was polymerized with PECA nanoparticles. The MS spectrum showed signals for the doubly charged (m/z 2904.4 for $z = 2$ $[M + 2H]^{2+}$) and singly charged (m/z 5805.1 for $z = 1$ $[M + H]^+$) insulin and an independent series of peaks with characteristic peak increments of 125 mass units over the mass range m/z 700-6000 for PECA polymers (Fig. **1d**). Peaks corresponding to co-polymers

$[M + (125)_n + H]^+$ were absent indicating that no residue interfered in the polymerization process. Similarly, no co-polymers were obtained when insulin was polymerized with butylcyanoacrylate nanoparticles (data not shown).

Fig. (3)

3.1.2. Non-histidine-containing peptides with ethyl-and butylcyanoacrylate (h-ACTH (18-39), IGF-1 (1-3))

The non-histidine containing peptide h-ACTH (18-39) did not co-polymerize with PECA. The MS spectrum of h-ACTH (18-39) polymerized with PECA nanoparticles showed a clear signal of the parent molecule at m/z 2465.6 $[M + H]^+$ (Fig. **3a**) and an independent series of PECA polymer peaks with characteristic peak increments of 125 mass units over the mass range m/z 1000-4000 (Fig. **3a**). Peaks corresponding to co-polymers $[M + (125)_n + H]^+$ were absent. Similarly, no co-polymers were obtained when h-ACTH (18-39) polymerized with butylcyanoacrylate (data not shown).

Interestingly IGF-1 (1-3), another non-histidine containing peptide co-polymerized with PECA indicated by a series of signals at m/z 802.3, 927.4, 1052.4 and 1177.5 $[301.3 +(125)_{4-7} + H]^+$ corresponding to adducts of polymer subunits $(125)_{4-7}$ to the parent mass of IGF-1(1-3)

$[301.3 + H]^+$ (Fig. **3b**). CID-MS/MS on IGF-1(1-3)/PECA co-polymers (*) $[301.3 + (125)_{4\text{-}6} + H]^+$ revealed clear signals of unmodified b2-ions (Gly-Pro) at m/z 155.1 for all three precursors selected (Fig. **4a-c**). The modification was located on the y-ion series as indicated by y1-and y2-ions shifted by $(125)_{4\text{-}6}$ mass units (Fig. **4a-c**).

Fig. (4)

3.2. Influence of pH on co-polymerization of peptides

We investigated whether a very low pH may impede the co-polymerization of D-Lys^6-GnRH and IGF-1 (1-3) with PECA during the *in situ* interfacial polymerization of PACA nanoparticles. Both peptides co-polymerized with PECA at pH 1.9 as indicated by MS-signals at $[1252.6 + (125)_{2\text{-}6} + H]^+$ for D-Lys^6-GnRH/PECA co-polymers (Fig. **5a**) and $[301.3 + (125)_{4\text{-}7} + H]^+$ for IGF-1 (1-3)/PECA co-polymers (Fig. **5b**). CID-MS/MS on both peptide co-polymers confirmed that the same residues were involved in the co-polymerization at pH 6.3 and pH 1.9 (data not shown).

Fig. (5)

4. DISCUSSION

Here we demonstrate that the small, histidine-containing peptides D-Lys^6-GnRH and [Asn^1, Val^5]-angiotensin II co-polymerize with both ethyl- and butylcyanoacrylate (Fig. **1a-c**), which confirms our previous findings of covalent interaction of His2 of D-Lys^6-GnRH with ethylcyanoacrylate [12]. In both peptides, we identified the histidine residue as the site of modification (Fig. **2**). The longer and slightly bulkier butyl-ester side chain of the butyl-monomer did not impair the ability of the nucleophilic peptide to attack the acrylate ethylene-group of the monomer. Protonation of the nucleophilic histidine at pH 1.9 also did not impede co-polymerization of D-Lys^6-GnRH with PECA nanoparticles (Fig. **5a**). Although more protonated, hence less nucleophilic histidine residues (pKa 6.04) are present at pH 1.9, the few remaining were reactive enough to initiate the polymerization reaction (zwitter-ionic pathway). A rapid elongation and termination reaction yields insoluble peptide/polymer co-polymers, which precipitate and aggregate to form nanoparticles. This results according to Le Chatelier's principle, in a shift of the equilibrium between protonated and unprotonated histidine, re-generating new unprotonated nucleophiles maintaining the reaction until completion.

The mere presence of histidine as a nucleophile alone, however, is not sufficient to initiate co-polymerization. From this study, the sterical availability of the nucleophile seems to be another important key factor to influence on the reaction, since insulin despite the presence of 2 histidine residues (His^{B5} and His^{B10}) did not co-polymerize (Fig. **1d**).

Fig. (6)

Insulin with 51 amino acid residues is a larger and more complex molecule compared to D-Lys^{6}-GnRH and [Asn^{1}, Val^{5}]-angiotensin II. In the literature, the three dimensional arrangement and folding of insulin is extensively discussed considering various aspects that may influence on the confirmation of insulin [19-22]. Depending on the insulin concentration, pH and presence of ligands, insulin shows a tendency to self-associate into insulin dimers or hexamers [19, 20]. The influence of phenols, such as m-cresol, is mainly discussed in the context of hexameric insulin, which forms in the presence of divalent cations, such as zinc. Zinc, however, is not present in our current study. We suggest that under polymerization conditions (pH 7.4, m-cresol, high surfactant concentration) the protein is most likely to persist in a dimeric self-association state [19, 23, 24]. For the formation of a dimer, the extended C-terminus of the B-chain (B24 - B30) engages in intermolecular hydrophobic interactions forming a two-stranded anti-parallel β-sheet [20, 21, 24] (Fig. **6**). However, the presence of phenols, such as m-cresol, may affect the confirmation of the N-terminus of the B-chain, which is the location of the histidine residues in question (His^{B5} and His^{B10}) [18, 20]. The extended B1-B9 strand may undergo a coil-to-helix conversion (Fig. **6**), which would leave the histidine residues buried within the phenolic pockets [19].

The h-ACTH fragment (18-39) that was used as a histidine-free peptide (control) did not co-polymerize with ethyl- and butylcyanoacrylate, as expected (Fig. **3a**). Interestingly the small histidine-free IGF-1 (1-3) fragment Gly-Pro-Glu did co-polymerize with both PECA (Fig. **3b,** Fig. **4**) and PBCA (data not shown). The C-terminal glutamic acid was identified as the site of interaction in IGF-1(1-3), which suggests an anionic reaction pathway. It is unclear whether the modification occurs at the C1 carboxyl-end or the carboxyl-group of the side chain. Neither internal glutamic acids such as Glu^{11}, Glu^{13}, Glu^{16}, and Glu^{21} in h-ACTH (18-39) nor free C-terminal carboxyl-groups in [Asn^1, Val^5]-angiotensin II and h-ACTH (18-39) were identified as sites of modification by acrylates in our experiments, suggesting that in IGF-1(1-3) the C-terminal position of Glu provokes reactivity towards PECA and PBCA. Glutamic acid has a pKa_1 of 2.1 for the C1 carboxylic acid and a pKa_2 of 4.07 for the side chain carboxylic acid. Controlling the pH in the polymerization medium should have enabled selective deprotonation, hence activation, of either carboxylic acid. In our studies, however, lowering the pH of the polymerization medium to pH 1.9 below the pKa_1 of glutamic acid did not prevent the co-polymerization of glutamic acid with PACA (Fig. **5b**). Internal glutamic acids compared to C-terminal glutamic acids such as Glu^{11}, Glu^{13}, Glu^{16}, and Glu^{21} in h-ACTH (18-39) were not modified. This could be an indication that only C-terminal glutamic acids react, which in turn would suggest the C1 carboxylic acid to be the site of modification. H^1-NMR studies could be employed to clarify the exact site of modification within the glutamic acid residue. Other free C-termini such as phenylalanine (Phe^{22} in h-ACTH (18-39)) or threonine (Thr^{B30} in insulin), however, seem less reactive towards PACA in an *in situ* polymerization as

compared to the C-terminal glutamic acid Glu^3 in IGF-1 (1-3) or strong nucleophiles, such as the histidine residues in D-Lys^6-GnRH, [Asn^1, Val^5]-angiotensin II.

The outcome of this study has various implications on applications using PACA nanoparticles. An expert opinion article by Graf *et al.* [25] recently pointed out the problems potentially associated with PACA nanoparticles due to their high variability in entrapment, release, and covalent interference of protein and peptide drugs in the polymerization process. The need to overcome those challenges and to optimize and control critical formulation parameters is mandatory for the successful introduction of PACA nanoparticles to the pharmaceutical market [25]. This present study is further raising concern about (i) the potential of various amino acid residues to co-polymerize with PACA nanoparticles and (ii) the little control there may be over the extent of co-polymerization by adjusting parameters such as the pH. For co-polymerized formulations, every co-polymer comprises a new potentially bioactive compound and requires full characterization and FDA approval/registration etc. Non-co-polymerized pharmaceutical formulations containing PACA need to be screened using technology such as MALDI TOF mass spectrometry to exclude co-polymerization and to verify their safety and suitability for the pharmaceutical market. For those systems, however, additional *in vivo* studies would have to demonstrate the absence of co-polymerization in the abundance of endogenous, physiological proteins and peptides.

5. CONCLUSIONS

The covalent interference of protein and peptide drugs depends on more than one key factor. Interference may take place via anionic or zwitter-ionic pathways involving amino acid residues such as histidine (zwitter-ionic pathway) or C-terminal glutamic acid (anionic pathway). The likelihood of interference seems highly dependant on the complexity and size of the molecule and the sterical availability of the reactive site. Tandem MALDI TOF/TOF mass spectrometry is an ideal technology for the characterization of components of PACA nanoparticles and identification of covalent drug/polymer interactions on the molecular level. Co-polymerization of peptide drugs with PACA is a phenomenon to be cognizant of when designing pharmaceutical delivery systems and the ability to reliably detect co-polymerization and/or chemical modifications of protein and peptide drugs is imperative for formulation scientists.

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