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On-line capillary electrophoresis-based enzymatic methodology for the study of polymer-drug conjugates☆

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ABSTRACT

This work aims at studying the potentialities of an on-line capillary electrophoresis (CE)-based digestion methodology for evaluating polymer–drug conjugates degradability in the presence of free trypsin (in-solution digestion). A sandwich plugs injection scheme with transverse diffusion of laminar profile (TDLFP) mode was used to achieve on-line digestions. Electrophoretic separation conditions were established using poly-L-Lysine (PLL) as reference substrate. Comparison with off-line digestion was carried out to demonstrate the feasibility of the proposed methodology. The applicability of the on-line CE-based digestion methodology was evaluated for two PLL-drug conjugates and for the four first generations of dendrigraft of lysine (DGL). Different electrophoretic profiles presenting the formation of di, tri, and tetralysine were observed for PLL-drug and DGL. These findings are in good agreement with the nature of the linker used to link the drug to PLL structure and the predicted degradability of DGL. The present on-line methodology applicability was also successfully proven for protein conjugates hydrolysis. In summary, the described methodology provides a powerful tool for the rapid study of biodegradable polymers.

1. Introduction

In the pharmaceutical industry, biodegradable polymers are the focus of attention particularly in the treatment of autoimmune diseases and cancer [1–4]. Due to the biodegradable polymers architecture, these macromolecules exhibit specific drug trapping and releasing properties with expected few side-effects. Conjugation of a low molecular weight drug to biodegradable polymers has been also proposed to improve the therapeutic index of small drug [4]. The resulting polymeric prodrug (called also polymer-drug conjugate PDC) helps in both enhancing the drug bioavailability and in preserving the drug activity during circulation, with minored immunological body response. In this context, PDCs composed of α- amino acids (α –AAs) are considered as promising representatives of synthetic biopolymers since after biodegradation the release products are essentially α–AAs, peptides and their derivatives. Thus, these poly(aminoacid)s-drug conjugates that are 1/ synthesized in a relatively simple manner 2/ cost-effective and 3/ present entire elimination post-treatment, have a considerable potential for drug delivery [1–4].

In this article, molecules of study were poly-L-Lysine-drug conjugates with linear (poly-L-Lysine: PLL) or dendritic architectures. The corresponding PDCs vary with respect to molecular weight, drug grafting, and resulting conformation. In addition, after absorption, the stability of the PDC and the rate of drug release directly impact its efficacy and safety. Consequently, it is a challenging task to manufacture reproducible PDC lots on large scale. Knowledge about drug-related impurities, PDC heterogeneity between lots, and percentage of drug grafting are requested to allow marketing authorization of the PDCs [5]. However, due to the inherent structural complexity of the PDCs, these characteristics remain challenging to obtain. There is a need for the development of validated techniques for the analysis of these multi-component mixtures and for establishing preclinical safety of PDCs. In choosing analytical techniques for quality control of PDCs, several factors are especially important: the methodology should be simple, rapid and developed with commercially available instruments.

A possible approach for controlling PDC composition and drug grafting consists in performing an enzymatic hydrolysis of the PDC to produce a mixture of surrogate peptides and amino acids (named digest) that are further analyzed by appropriate separation

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methods. To date, the enzymatic digestion step of polypeptides (to a lesser extend for poly(amino acids)) has been performed off-line with protease using mostly two-dimensional gel electrophoresis and high performance liquid chromatography (HPLC) with mass spectrometry (MS) in order to structurally characterize polypeptide biopharmaceuticals or to apply proteomic bottom-up approaches [6]. However, the off-line digestion step is both time and reactants consuming, and often show cross contaminations by endogenous compounds that further impair the quality of the analysis [7]. One first alternative could be to use capillary electrophoresis (CE) in combination with immobilized enzyme reactors (IMERs) to achieve on-line enzyme assay [8] but this is limited due to the loss of enzyme activity upon immobilization, the cost and the restricted particles size of the support commercially available [7]. A second alternative approach has been proposed by Li et al. This approach consists in the use of two-dimensional CE system employing a replaceable monolithic reactor for on-line protein digestion [9]. This two-dimensional CE system with MS detection helped to improve protein identification scores. Unfortunately, this system has the same disadvantages of IMERs and is not commercially available. Consequently, this cannot be implemented for PDCs quality control. All of the above approaches have been established for qualitative studies aiming to identify proteins by MS via peptide fragment identification after proteolysis. On the opposite, quality control of PDC requires both qualitative and quantitative studies to estimate lot purity and drug grafting.

In this paper, we present an automated on-line CE methodology employing enzyme in solution for hydrolyzing dendrigraft of lysine (DGL) and PLL-drug conjugates, in which the silica capillary is used both as nano-enzymatic reactor and separation support. Enzymes in solution are in their most active format and are easy to handle. Moreover, conventional on-line enzyme assays are usually developed for the screening of pharmaceuticals (drugs having low-molecular-weight), the evaluation of enzyme activity and the studying of drug–drug interactions. In this paper, the on-line enzyme assays are applied to DGL and PLL-drug conjugates (substrates having molecular weights above 5 kDa) using trypsin as model enzyme to study PDCs proteolysis. The reactants are injected sequentially; the mixing of reactant plugs is done by transverse diffusion, which is the first application in case of proteolysis. The separation is done by applying an electric field using conventional CE instrument. The developed methodology is rapid, cost- and time-saving. We found out that DGL and PLL-drug conjugates proteolysis is highly repeatable. In addition, we demonstrated that this on-line trypsin digestion CE methodology can be easily applied to protein conjugates.

2. Materials and methods

2.1. Chemicals

Native Poly-L-Lysine (PLL, 15–30 kDa), PLL-drug conjugates linking taurine (Tau), Bovine Serum Albumin (BSA) and its conjugate bearing indole-3-acetic acid (IAA) named BSA-IAA were supplied in lyophilized form by GemacBio (France, http://www.gemacpharma.com).

Dendrigrafts of poly L-Lysine (DGL, generations G_1, G_2, G_3, G_4), were gratefully provided by Colcom (France, http://www.colcom.eu) and used as received.

Sequencing grade modified trypsin, citric acid (≥ 99.5%), 6-aminohexanoic acid (ε-amino caproic acid, ≥ 98.5%), sodium hydroxide (≥ 98%), hydrochloride acid (≥ 37%), ammonium bicarbonate (≥ 99.5%), phosphoric acid (≥ 85%), dilysine (Lys2), trilysine (Lys3), tetralysine (Lys4) and pentalysine (Lys5), and poly(ethylene oxide) (PEO) of molecular weight 1,000,000 were purchased from Sigma–Aldrich (France). Imidazole (≥ 99%) was bought from Acros Organics (France). All aqueous solutions were prepared with a Milli-Q purification system from Millipore (France). The fused silica capillaries were purchased from Polymicro Technologies (United States).

2.2. Capillary electrophoresis (CE)

A PA800 system (Beckman, Fullerton, USA) was used for CE analysis. The capillaries (i.d. 50 μm, total length 50 cm, length to the detector 40 cm) were coated according to the standard procedure described elsewhere [10]. Briefly, the capillaries were preconditioned with 1 M NaOH, for 20 min followed by 10 min flushing with Milli-Q water under 20 psi.

In between runs, the capillary was successively rinsed with 1 N NaOH, Milli-Q water, 0.1 M HCl (5 min each under 20 psi), and finally with coating solution for 10 min under 20 psi. The coating solution is composed of acidified polyethylene oxide (PEO) at 0.2% (m/v) in 0.1 M HCl. The capillary was thoroughly flushed (20 psi, 5 min) with the background electrolyte (BGE) before introducing nanovolumes (plugs) of enzyme and its substrates. The injections were performed by hydrodynamic pressure at the anodic end of the capillary.

The BGE was composed of a mixture of citric acid (27 mM) and ε-amino caproic acid (207 mM) mixture at pH 5.0. Its ionic strength (I) was 75 mM. This buffer was used to separate lysine oligomers from others hydrolysis products, remaining polymeric substrates, and enzyme. For the study of trypsin digestion of BSA and BSA-IAA, a BGE composed of phosphate buffer (pH 2.5, I 50 mM) was used.

A voltage of 15 kV was applied to separate the substrate, the enzyme and the hydrolysis products. The capillary was thermostated at 25 °C during the whole procedure. UV detection was performed at 214 nm. 5 μL of imidazole solution at 0.25 g/L (or 5.0 g/L) was added to 95 μL of the solution to be analyzed.

2.3. Off-line tryptic digestion

The off-line digestion procedure was composed of two steps: the substrate was first incubated with enzyme in a vial for a certain period of time. Then several nL of the sample digest were injected at the inlet of the PEO-coated capillary. The substrates PLL, PLL-drug conjugates and DGLs were exactly prepared at 1 g/L in Milli-Q water. A trypsin stock solution was prepared by dissolving 20 μg of trypsin in 100 μL of 5 mM HCl. 20 μL of trypsin stock solution were added to the PDC solution to have an enzyme-to-substrate ratio (E/S) of 1/20 (w/w) in proteolytic buffer composed of 100 mM ammonium bicarbonate (pH 8.3, I 100 mM). Subsequent to the trypsin addition, the mixture was incubated in a dry bath at 37 °C. The tryptic digestion was stopped by freezing the sample in liquid nitrogen. The frozen samples were stored at −20 °C for a short period of time before analysis.

2.4. On-line tryptic digestion

On-line tryptic digestion was achieved in a PEO-coated capillary. The substrates were exactly prepared at 1 g/L in Milli-Q water. A trypsin solution was prepared at 0.2 g/L. For proteins, aliquots were denatured at 80 °C for 10 min in micro-centrifuge tubes prior to injection. All the substrates **S** (DGL and PLL derivatives, BSA and BSA-IAA), the trypsin (enzyme abbreviated by **E**), and the proteolytic buffer (symbolized by "**PB**") were introduced hydrodynamically into the capillary. PB was composed of 100 mM ammonium bicarbonate (pH 8.3, I 100 mM). Unless stated otherwise, volumes of S, E, and PB plugs were 6.65 nL, 3.56 nL and 1.42 nL, respectively. Reactants mixing and digestion inside the capillary were achieved by transverse diffusion as described by Krylov group