Capillary electrophoresis-based assessment of nanobody affinity and purity

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HIGHLIGHTS

- ACE is used to probe the affinity between nanobody and receptor.
- Coatings allow precise determination of effective mobility shifts.
- Nanomolar K_d values of several separated components were obtained simultaneously.
- CE–MS of nanobody allows identification of separated components.
- Modifications do not alter the affinity of nanobody towards receptor.

GRAPHICAL ABSTRACT
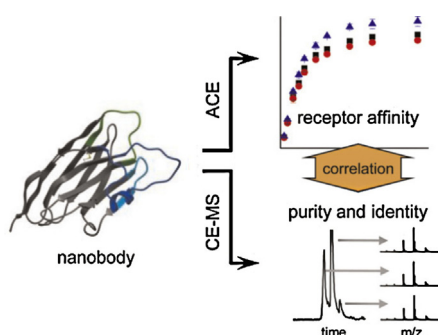
ABSTRACT

Drug purity and affinity are essential attributes during development and production of therapeutic proteins. In this work, capillary electrophoresis (CE) was used to determine both the affinity and composition of the biotechnologically produced "nanobody" EGa1, the binding fragment of a heavy-chain-only antibody. EGa1 is an antagonist of the epidermal growth factor receptor (EGFR), which is overexpressed on the surface of tumor cells. Using a background electrolyte (BGE) of 50 mM sodium phosphate (pH 8.0) in combination with a polybrene-poly(vinylsulfonic acid) capillary coating, CE analysis of EGa1 showed the presence of at least three components. Affinity of the EGa1 components towards the extracellular domain of EGFR was assessed by adding different concentrations (0–12 nM) of the receptor to the BGE while measuring the effective electrophoretic mobility of the respective EGa1 components. Binding curves obtained by plotting electrophoretic mobility shifts as a function of receptor concentration, yielded dissociation constants (K_d) of 1.65, 1.67, and 1.75 nM for the three components, respectively; these values were comparable to the K_d of 2.1 nM obtained for the bulk EGa1 product using a cellular assay. CE with mass spectrometry (MS) detection using a BGE of 25 mM ammonium acetate (pH 8.0) revealed that the EGa1 sample comprised of significant amounts of deamidated, bisdeamidated and N-terminal pyroglutamic acid products. CE–MS using a BGE of 100 mM acetic acid (pH 2.8) in combination with a polybrene–dextran sulfate–polybrene capillary coating demonstrated the additional presence of minor

products related to incomplete removal of the signal peptide from the produced nanobody. Combining the results obtained from affinity CE and CE-MS, it is concluded that the EGa1 nanobody product is heterogeneous, comprising highly-related proteins that exhibit very similar affinity towards EGFR.

1. Introduction

The epidermal growth factor receptor (EGFR) is a membrane-bound receptor that, upon ligand binding, stimulates intracellular protein-tyrosine kinase activity. This initiates several signal transduction cascades, leading to DNA synthesis and cell proliferation. Upregulation of the EGFR is implicated in a number of human cancers, including colorectal, lung, brain, and head and neck tumors. Consequently, EGFR is a recognized target for anti-cancer therapies [1]. Over the past years, increasing interest has arisen for so-called nanobodies as a novel approach to antagonize EGFR [2–5]. Nanobodies are small antibody fragments derived from heavy-chain-only antibodies of camelids and possess attractive characteristics, like high chemical stability and nanomolar affinity [6]. The nanobody EGa1 has shown to be an antagonist for the EGFR with good potential for therapeutic use [4,5].

Nanobodies, like EGa1, are expressed in and isolated from *Escherichia coli*. During the biotechnological production process, which may show variability, product diversity, isoforms and closely-related degradation products may be introduced [7,8]. Some protein modifications may have no consequences for activity or toxicity, whereas other variants could have adverse biological and pharmacological effects [9,10]. Therefore, besides compositional characterization, there is a need for analytical methodologies enabling bioactivity assessment of the modified species next to the parent protein drug.

In the pharmaceutical field many techniques, such as enzyme-linked immunosorbent assays, surface plasmon resonance, and nuclear magnetic resonance, are applied for affinity determinations of ligands towards, for example, enzymes and receptors [11–13]. Most affinity assays only provide overall information on the bulk product; that is, they cannot distinguish between the multiple protein components frequently present in biopharmaceutical products. In this respect, affinity capillary electrophoresis (ACE) is an interesting technique potentially allowing efficient separation of compounds while simultaneously enabling determination of their affinity [12–14]. Affinity measurements by CE are based on the fact that protein–ligand binding causes a change in the electrophoretic mobility of the protein and/or ligand [12,15]. Depending on the kinetics of the affinity interaction, these differences in electrophoretic mobility can be exploited in two different ways. When kinetics are slow, the free protein or ligand can be discretely separated from the protein–ligand complex present in a preequilibrated mixture. When interaction kinetics are fast, the gradual shift of the electrophoretic mobility of protein or ligand can be monitored as a function of ligand/protein concentrations present in the capillary during analysis.

ACE has shown very useful for the simultaneous determination of affinities of low molecular weight ligands towards a binding protein [15–18]. However, ACE has been applied only occasionally for the assessment of protein–protein interactions [19–24]. Moreover, these studies predominantly deal with the interaction between antibodies and proteinaceous antigens, establishing sub-nanomolar to micromolar dissociation constants. So far, ACE has not been used for probing of protein–receptor binding. In addition, the simultaneous assessment of the affinities of a proteinaceous parent drug and its isoforms and/or degradation products has not been studied by ACE before. In some ACE studies, protein isoforms or components have been observed [19,20], but their affinity was not determined, and also their identity often remained elusive.

The interaction between bulk EGa1 and EGFR has been studied by cellular assays and surface plasmon resonance [4,5], but no distinction between EGa1 components (*e.g.* isoforms and/or impurities) was made. In the present study, an ACE method employing UV detection was developed for the assessment of the affinity between EGFR and the EGa1 nanobody, including its production modifications. In addition, CE with mass spectrometric detection (CE–MS) was performed to confirm and establish the identity of EGa1 and its product-related components. We have studied some (modified) nanobodies with CE-UV and CE–MS before [3,25,26]. In these studies, noncovalent capillary coatings were employed to prevent protein–capillary wall interactions, to allow efficient protein separations, and/or to reliably determine ligand-binding. In order to achieve efficient ACE and CE–MS of EGa1 in the present study, separation conditions were optimized to permit separations in a quasi-physiological environment and allow construction of binding isotherms, yielding dissociation constants for all EGa1 components.

2. Materials and methods

2.1. Chemicals

Acetic acid (99.9%), ammonium hydroxide (25%), sodium hydroxide, phosphoric acid (85%), sodium dihydrogen phosphate, disodium hydrogen phosphate, and isopropanol were obtained from Merck (Darmstadt, Germany). Polybrene (hexadimethrine bromide; PB), poly (vinylsulfonic acid) (PVS), dextran sulfate (DS) sodium salt, and isopropanol were purchased from Sigma–Aldrich (Steinheim, Germany). Formamide was from Fluka (Steinheim, Germany). EGa1 was expressed and purified as described previously [4]. EGa1 was formulated in phosphate buffered saline (pH 7.4) and diluted prior to CE analysis with deionized water to the desired concentration. The pI of EGa1 was estimated based on its amino acid sequence (Fig. 1) using the calculator of the Expasy website [27]. ECD EGFR was obtained from R&D Systems (Oxon, UK) and was dissolved at a concentration of 0.1 mg mL^{-1} in phosphate buffered saline (pH 7.4). BGEs of 50 mM sodium phosphate (pH 3.0 and 8.0) were prepared by mixing equimolar solutions of either phosphoric acid and sodium dihydrogen phosphate or sodium dihydrogen phosphate and disodium hydrogen phosphate in the appropriate ratio to reach pH 3.0 and 8.0, respectively. A BGE of 25 mM ammonium acetate was prepared by dissolving 0.093 mL ammonium hydroxide in 50 mL deionized water and setting the pH with 10 times diluted glacial acetic acid. A BGE of 100 mM acetic

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MKYLLPTAAA GLLLLAAQPA MAQVQLQESG GGLVQPGGSL RLSCAASGRT FSSYAMGWFR QAPGKEREFV
AAIRWSGGYT YYTDSVKGRF TISRDNAKTT VYLQMNSLKP EDTAVYYCAA TYLSSDYSRY ALPQRPLDYD
YWGQGTQVTV SSAAAEQKLI SEEDLNGLEH HHHHH
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Fig. 1. Amino acid sequence of EGa1 (amino acids 23–175) including its signal peptide (amino acids 1–22; underlined).