Supplementary Information

Label-free quantification of antibody-oligonucleotide conjugates with varying stoichiometries using capillary zone electrophoresis

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Experiment

Materials. Rituximab (RTX) is provided by Shanghai Renji Hospital. Oligonucleotides modified with HS-SH C6, DBCO, and biotin were purchased from Sangon Biotech (China) with sequence (GTGGTAGTTAGGAGTGAATGC). Borate, HP-β-CD, and SDS were purchased from Sinopharm Chemical Reagent Co., Ltd..

Construction of Antibody-Oligonucleotide Conjugates

Covalent Conjugation 1. RTX was subjected to desalting using a 100 kDa protein concentrator (PES membrane, Thermo Scientific™) through centrifugal filtration. The buffer was subsequently exchanged with phosphate-buffered saline (PBS, pH 7.4). A 10 mM solution of Sulfo-SMCC (Thermo Scientific™) was prepared and added to RTX (10 mg/mL) at a molar ratio of 10:1 (Sulfo-SMCC: rituximab). The reaction was carried out at 4°C for 2 hours, followed by the removal of unreacted small molecules using a Zeba desalting column (Thermo Fisher Scientific). The thiol-modified oligonucleotide was then conjugated to the RTX at a molar ratio of 3:1 (oligonucleotide: RTX) and incubated overnight at 4°C.

Covalent Conjugation 2. Azide group**s** were modified on RTX using **t**he SiteClick™ Antibody Azide Modification Kit (Thermo Scientific™) according to the manufacturer's instructions. The N_3-modified RTX was then conjugated to DBCO-oligonucleotide at a molar ratio of 1:3 (oligonucleotide: RTX) and incubated overnight at 25°C.

Non-covalent Conjugation. RTX was biotinylated using the EZ-Link™ Sulfo-NHS-SS-Biotin kit (Thermo Scientific™) according to the manufacturer's instructions. The biotinylated RTX was subsequently mixed with NeutrAvidin (Thermo Scientific™) at a 1:1 molar ratio and incubated at room temperature for 1 hour. Following this, biotin-modified oligonucleotides were added to the RTX-NeutrAvidin conjugate at a molar ratio of 1:4 (oligonucleotide:antibody-NeutrAvidin) and incubated at room temperature for an additional hour.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) characterization of AOCs

SDS-PAGE was performed for AOC separation using a 10% resolving gel (pH 8.8) and a 5% stacking gel (pH 6.8). Electrophoresis was run at 90 V until the samples entered the resolving gel, then at 120-150 V until the bromophenol blue front reached the bottom. Gels were stained with Coomassie Brilliant Blue for 1 h and destained until bands were visible.

SDS-capillary gel electrophoresis (SDS-CGE) characterization of AOCs

SDS-CEG was performed on SCIEX PA 800 Plus Pharmaceutical Analysis System equipped with a UV detector and a fused-silica capillary (inner diameter: 50 μm, effective length: 20 cm total length: 30.2 cm). The capillary was coated with a polyacrylamide gel to enhance separation efficiency and stability. The running buffer consisted of a phosphate buffer (pH 8.8, 20 mM) containing 0.1% (w/v) polyacrylamide gel. Before injection, samples were diluted to a concentration of 10 mg/ml. The capillary was conditioned by flushing with 0.1 M NaOH for 5 minutes, followed by the running buffer for 10 minutes before each run. Electrophoresis was conducted at 25°C with an applied voltage of 15 kV. The injection time was set to 20 seconds at 5 kV.

Capillary zone electrophoresis (CZE) characterization of AOCs

CZE was performed using the SCIEX PA 800 Plus Pharmaceutical Analysis System. Analytes, including AOCs, RTX, and ODN, were each prepared at a concentration of 1 mg/mL in deionized water. Deionized water was also used as a blank control to ensure the absence of interfering signals. The electrophoresis conditions were optimized as follows: a diode array detector (DAD) was employed with full-wavelength scanning to monitor analyte migration. Separations were conducted using a fused silica capillary (50 μm inner diameter, 50 cm effective length, 60.2 cm total length). Sample injection was performed at 0.5 psi for 5 seconds, followed by the injection of separation buffer at 1 psi for 5 seconds. The separation voltage was set to 10 kV, and the total run time was 40 minutes. The capillary temperature was maintained at 25 °C, while the sample compartment was kept at 15 °C. The separation buffer was prepared by mixing 2.5 mL of 200 mM borate, 2.5 mL of 100 mM HP-β-CD, and 1 mL of 100 mM SDS, and then diluted with water to a final volume of 10 mL. Data acquisition was performed at a sampling rate of 2-4 Hz with a slit width of 100 × 200 μm.

High-performance liquid chromatography-mass spectrometry (HPLC-MS) characterization of AOCs

SCIEX X500B QTOF system was used for TOF-MS scanning. Chromatography and mass spectrometry conditions are as follows:

Chromatography

Phase A: 2% acetonitrile in water (0.1% formic acid)

Phase B: 98% acetonitrile in water (0.1% formic acid)

Column: Waters BEH200 SEC, 150 × 4.6 mm, 1.7 μm.

Elution Mode: Isocratic elution with 35% Phase B.

Flow Rate: 0.22 mL/min.

Column Temperature: 40 °C.

Mass Spectrometry

Parameters	Value
Curtain gas (psi):	35
Collision Gas(psi):	7
IonSpray Voltage(V):	5000
Temperature(°C):	450
Declustering Potential(V):	275
Ion Source Gas1(psi):	45
Ion Source Gas2(psi):	45
TOF start mass (m/z):	600
TOF stop mass (m/z):	5000
Accumulation time (s):	1

Table S1 Mass Spectrometry Parameters

Supplementary Figures

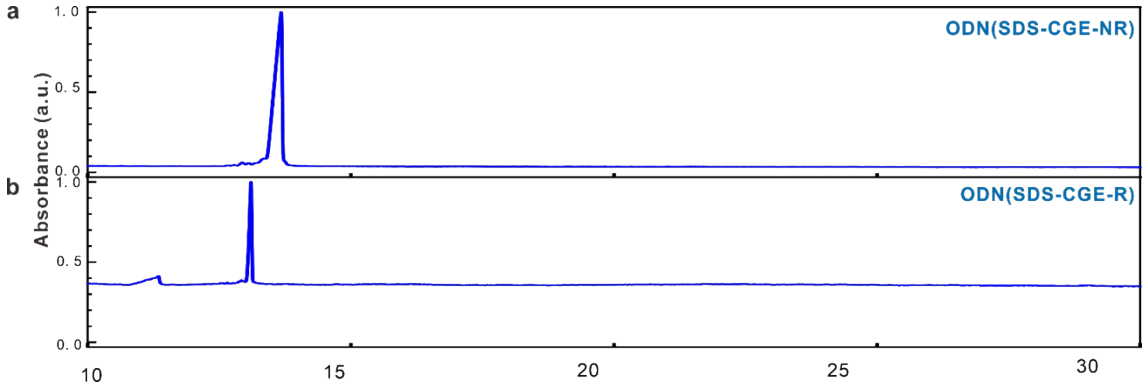

Fig. S1 Non-reducing (a) and reducing (b) SDS-CGE electropherogram of ODN.

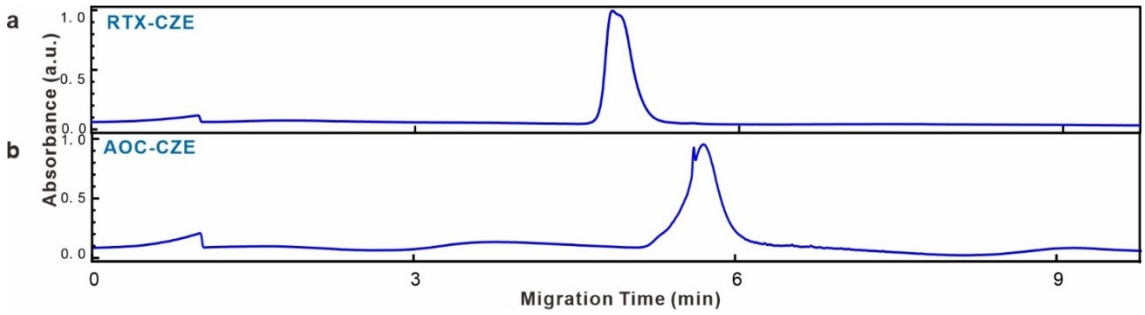

Fig.S2 CZE electropherograms of RTX (a) and AOC (b) in buffer at pH 3.

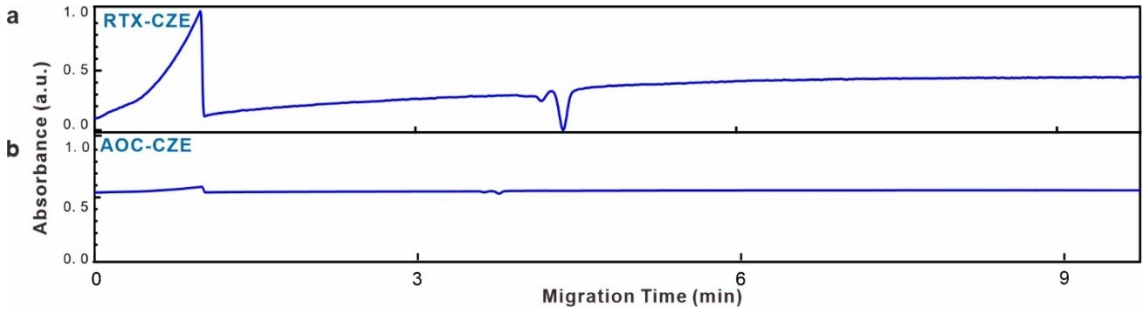

Fig.S3 CZE electropherograms of RTX (a) and AOC (b) in buffer at pH 5.5.

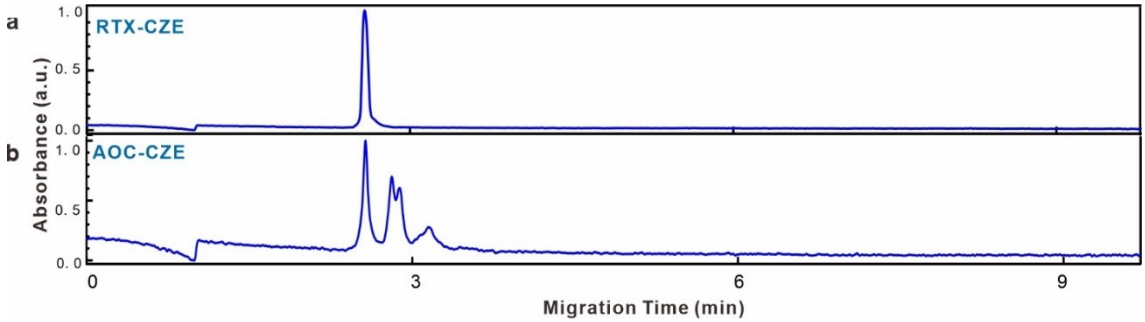

Fig.S4 CZE electropherograms of RTX (a) and AOC (b) in buffer at pH 7.5.

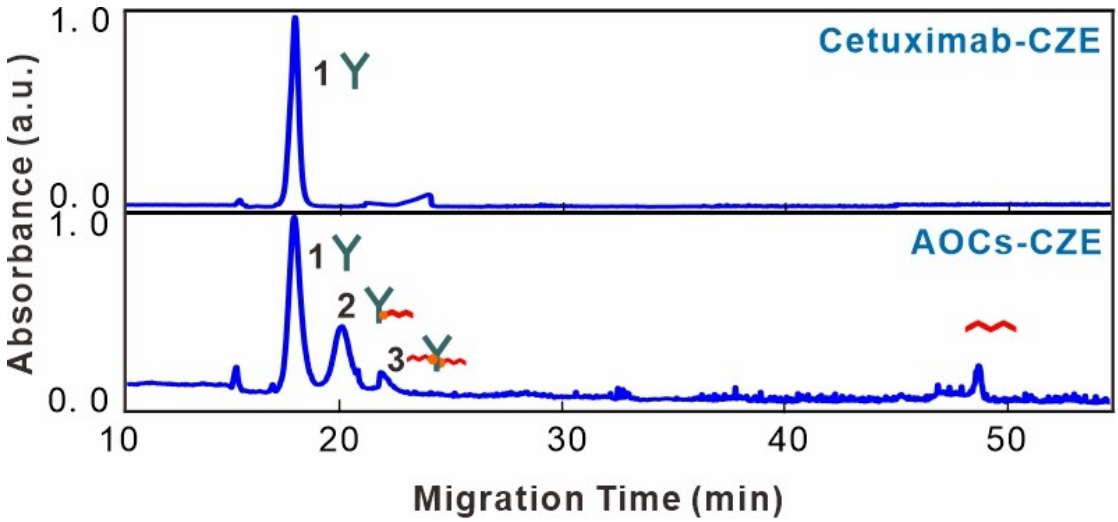

Fig.S5 CZE electropherograms of Cetuximab and its corresponding AOCs.