Characterization of Nimotuzumab Immunoconjugates

The purity of DOTA-nimotuzumab, DOTA-nimotuzumab-PEG_6-DM1-Low and DOTA-nimotuzumab-PEG_6-DM1-High immunoconjugates was determined using size exclusion HPLC (Waters 2796 Bioseparations Module, Waters 2487 Dual λ Absorbance Detector, XBridge® BEH 200A SEC 3.5 µm 7.8 x 300 mm column, Waters Corporation) in PBS at a flow rate of 0.6 mL/min. The molecular weight and purity was characterized using 2100 Bioanalyzer system (Agilent) following the manufacturer's protocol.

Mouse Xenograft Models

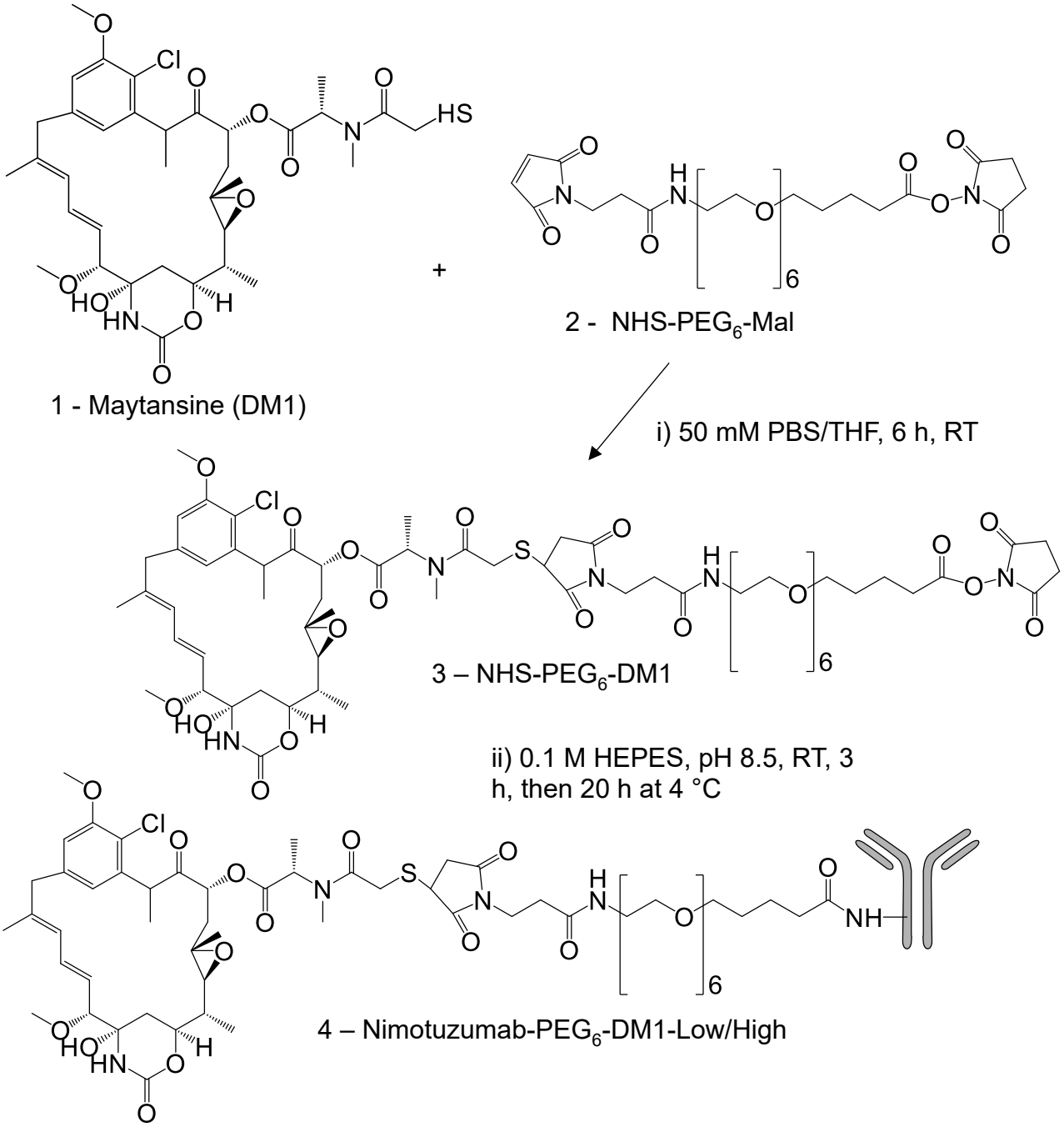
All animals used in *in vivo* experiments were cared for and maintained under the supervision and guidelines of the University of Saskatchewan Animal Care Committee (protocol # 20170084). Female CD-1 nude mice were obtained from Charles River Canada at 4 weeks of age and housed in a 12 h light, 12 h dark cycle in a temperature and humidity controlled vivarium. Animals had ad libitum access to mouse diet (Lab Diet) and water. After one week of acclimatization, mice were subcutaneously injected with a suspension of 5 – 10 x 10^6 DLD-1 cells in 100 µL of a 1:1 mixture of serum-free MEM/EBSS medium (HyClone Laboratories) and matrigel matrix basement membrane (Discovery Laboware) in the hind limb. Tumor growth was followed with caliper measurements.

Radiolabeling with ^{111}In and Stability Studies

For radiolabeling with $^{111}InCl_3$, 50 – 100 MBq of $^{111}InCl_3$ (Nordion) was incubated with 50 – 100 µg antibody in 0.1 M sodium acetate (pH 5.0) at room temperature for 90 min. The reaction was monitored using iTLC with 100 mM sodium citrate buffer (pH 5.0) and analyzed

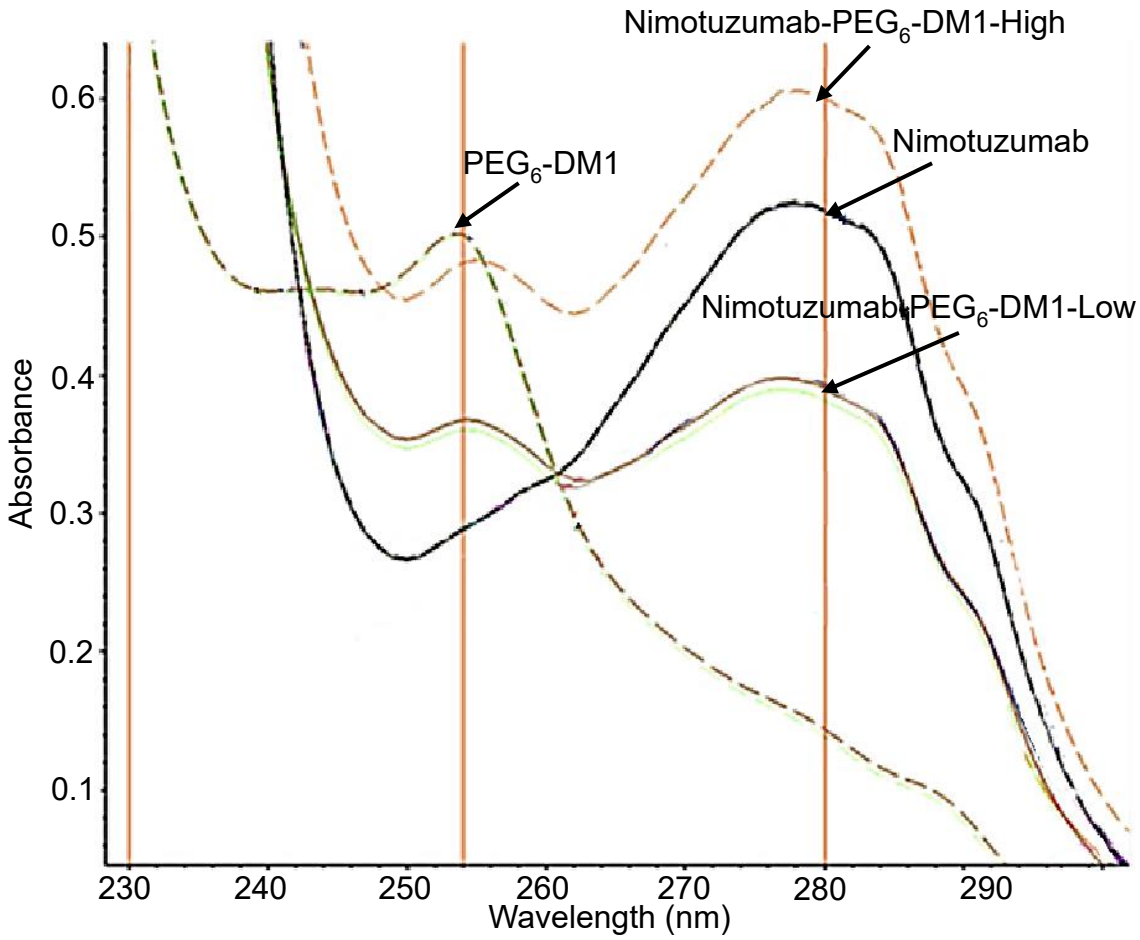
with ScanRam (LabLogic). After incubation, the ^{111}In-labeled conjugates were purified using centrifugal filters (10K, EMD Millipore) with PBS. The purity of the radioimmunoconjugates was determined using size exclusion radio-HPLC and iTLC as described earlier.

Stability was evaluated in human plasma and PBS. The radiolabeled compound was added to human plasma or PBS and incubated at 37 °C for up to 5 days (n = 3). Radiochemical purity was determined over time using iTLC. Dissociation of ^{111}In from the complex due to different metal ions (Cu^{2+}, Mg^{2+}, Zn^{2+} and Fe^{2+}) was measured by incubating with 1 M solutions of $CuSO_4$, $MgSO_4$, $ZnCl_2$ and $FeSO_4$ at 37 ºC for 5 days and monitored using iTLC.



SUPPLEMENTAL FIGURE 1. Synthetic scheme of nimotuzumab drug conjugation. Maytansine (DM1) was reacted with bifunctional linker NHS-PEG$_6$-Mal (2) in 50 mM PBS/THF for 6 h at room temperature to generate the NHS-PEG$_6$-DM1. NHS-PEG$_6$-DM1 was analyzed by mass spectrometry and NMR. Different fold excess of NHS-PEG$_6$-DM1 was then reacted with

nimotuzumab in 0.1 M HEPES pH 8.5 at room temperature for 3 h followed by 4 ºC for 20 h to yield nimotuzumab-PEG$_6$-DM1-Low (3 – 4 drugs per antibody) or nimotuzumab-PEG$_6$-DM1-High (7 – 8 drugs per antibody).



SUPPLEMENTAL FIGURE 2. Determination of Antibody-drug-ratio (UV method). Distinct UV spectra of drug, antibody and antibody drug conjugates are shown. Drug to antibody ratio was determined using UV method. Antibody and drug each has absorbance maxima at different wavelengths i.e. 280 and 254 nm respectively. Simultaneous equations were generated for both drug and antibody using the Beer–Lambert law. By solving the simultaneous equations, the number of drug per antibody was determined.

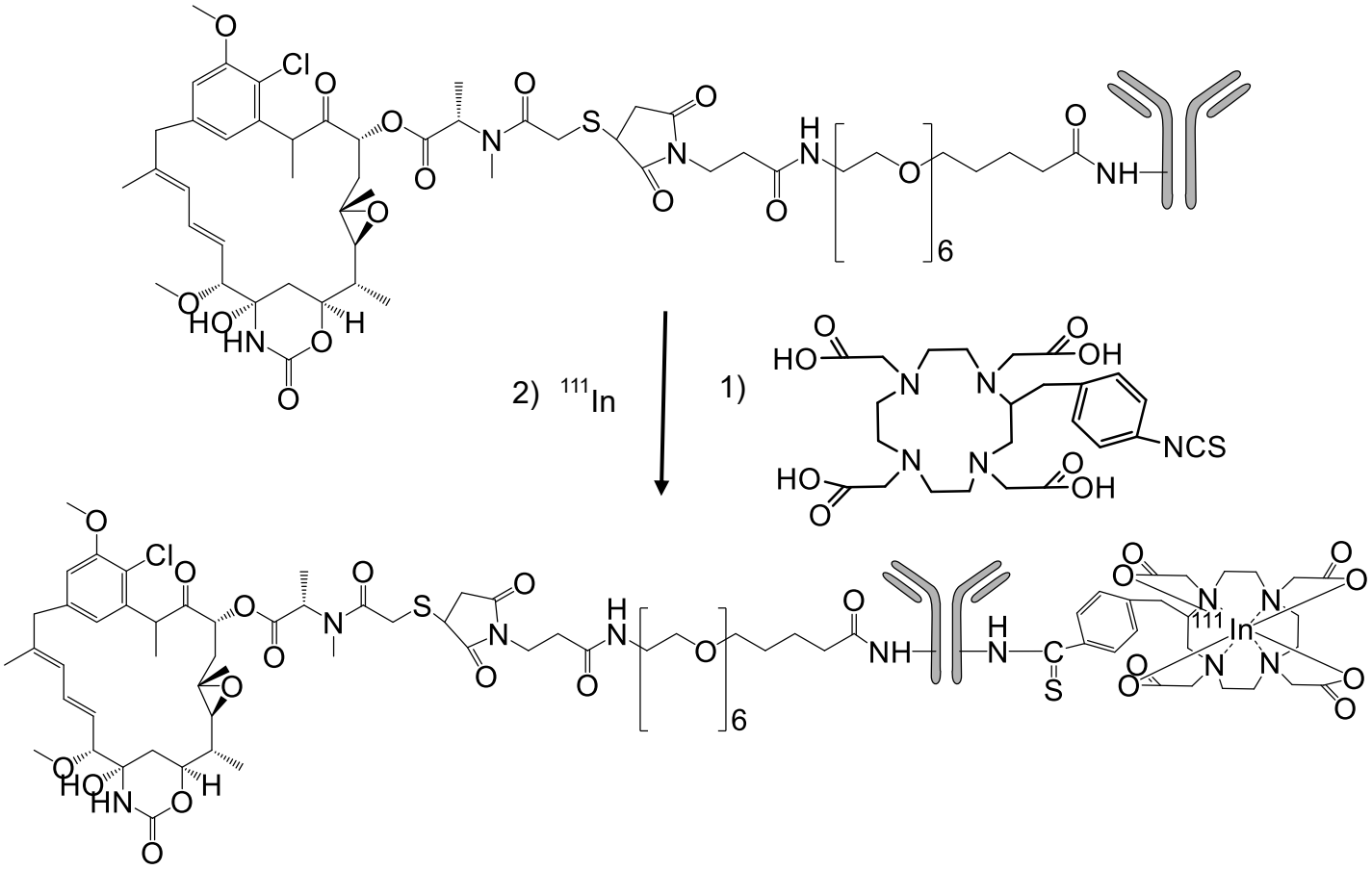
Equation 1: Antibody- Absorption at 280 nm

$$A_{280} = (\varepsilon_{drug}^{280} C_{drug} + \varepsilon_{mAb}^{280} C_{mAb}) l$$

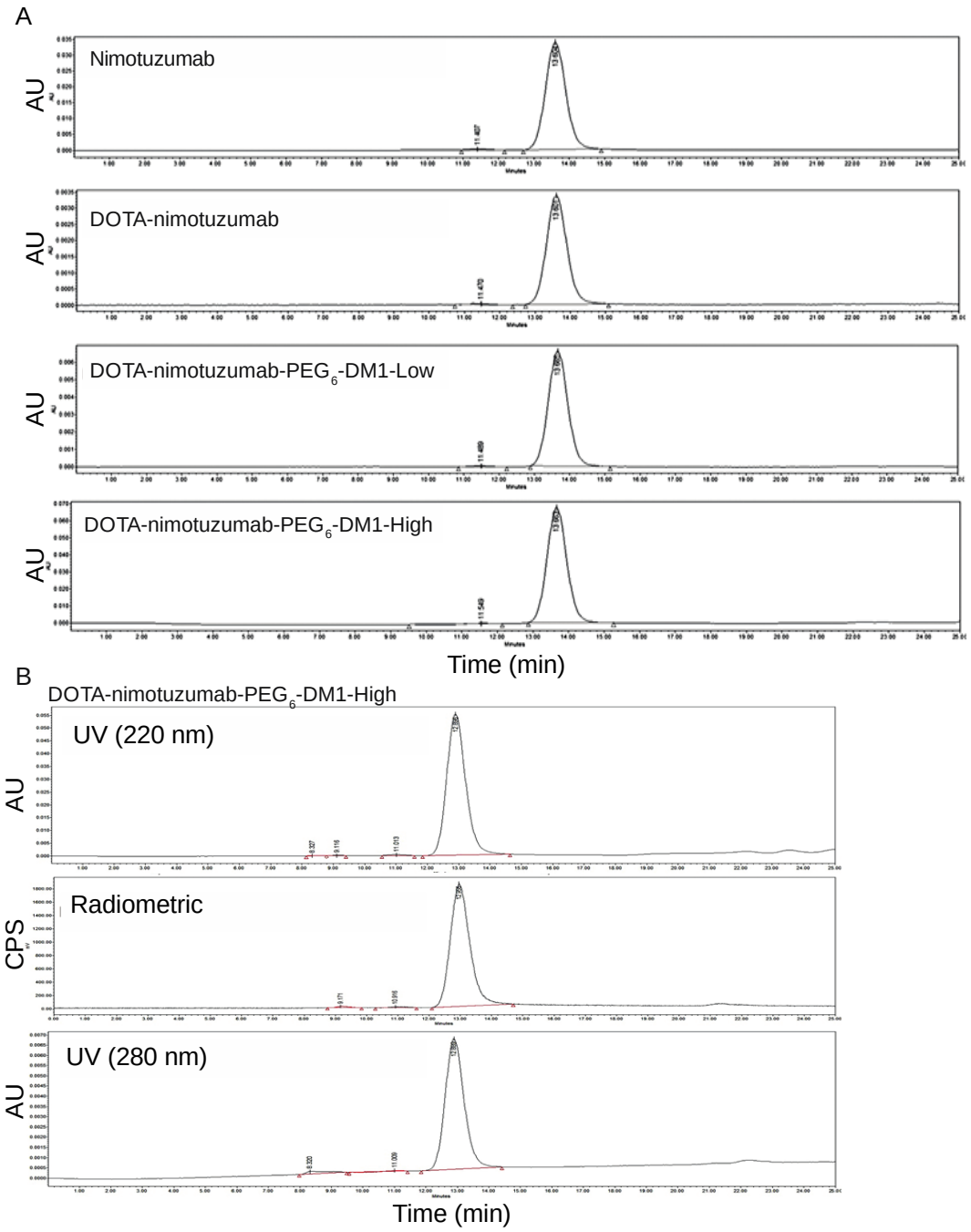
Equation 2: Antibody- Absorption at 254 nm

$$A_{254} = (\varepsilon_{drug}^{254} C_{drug} + \varepsilon_{mAb}^{254} C_{mAb}) l$$

The average drug to antibody ratio was determined by dividing the C_{drug} by C_{mab} and was expressed in moles of drug to moles of antibody.

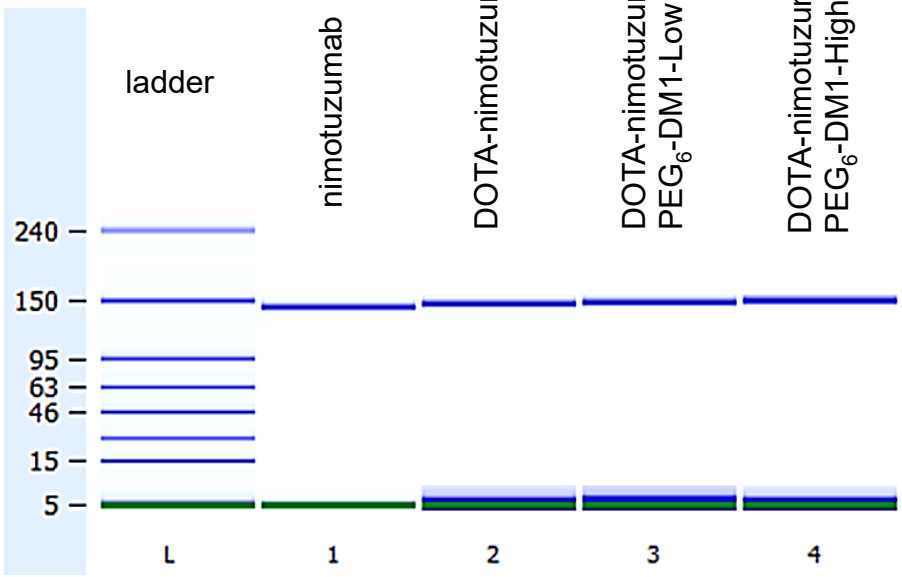


SUPPLEMENTAL FIGURE 3. Conjugation of DOTA and radiolabeling with ^{111}In.

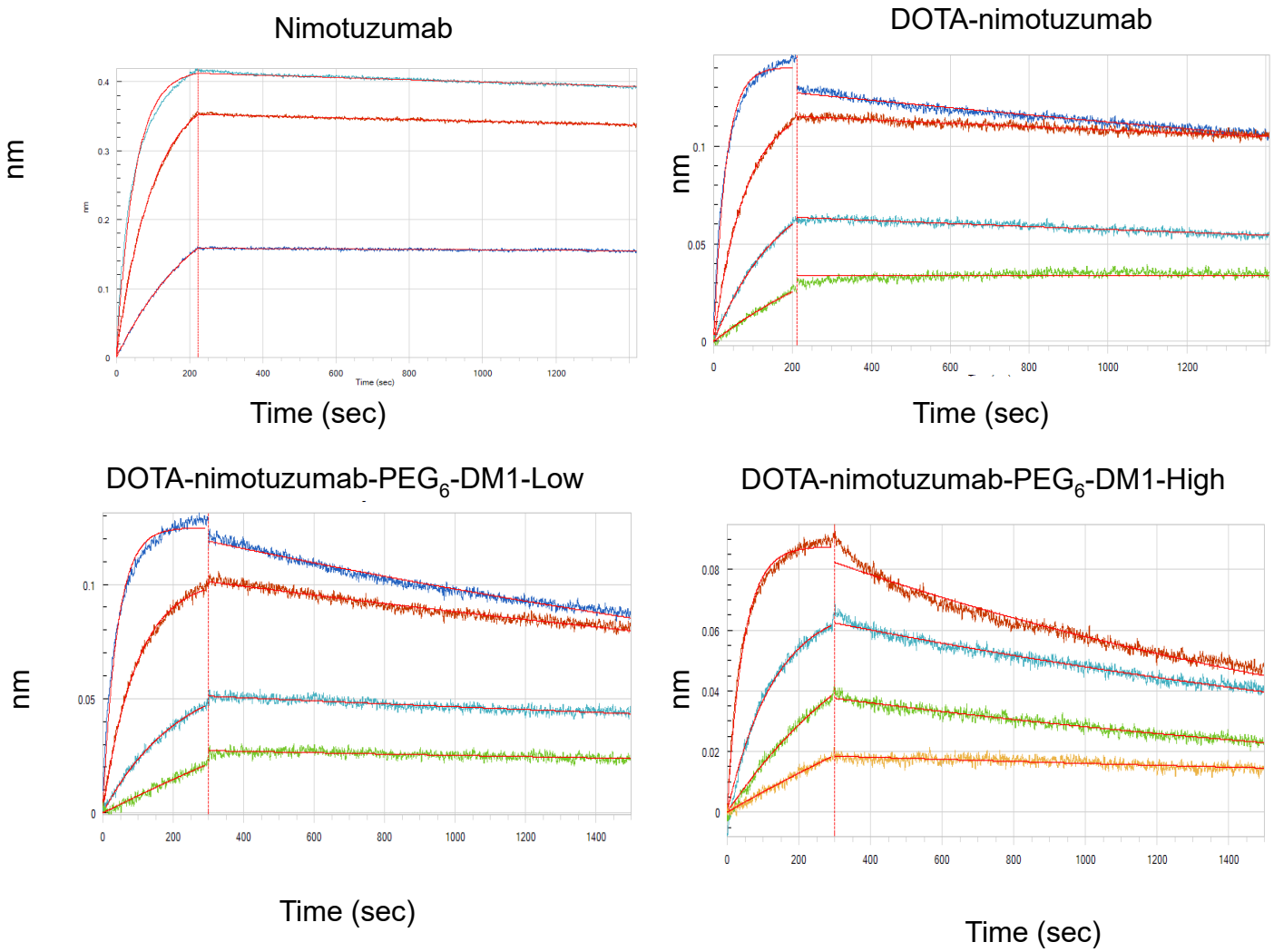


SUPPLEMENTAL FIGURE 4. Representative HPLC chromatograms of nimotuzumab, DOTA-nimotuzumab, DOTA-nimotuzumab-PEG6-DM1-Low and DOTA-nimotuzumab-PEG6-DM1-High at 280 nm. B): Representative HPLC chromatograms showing UV (@280 nm and 220 nm) and radiometric channels of ^{111}In-nimotuzumab-PEG6-DM1-Low.

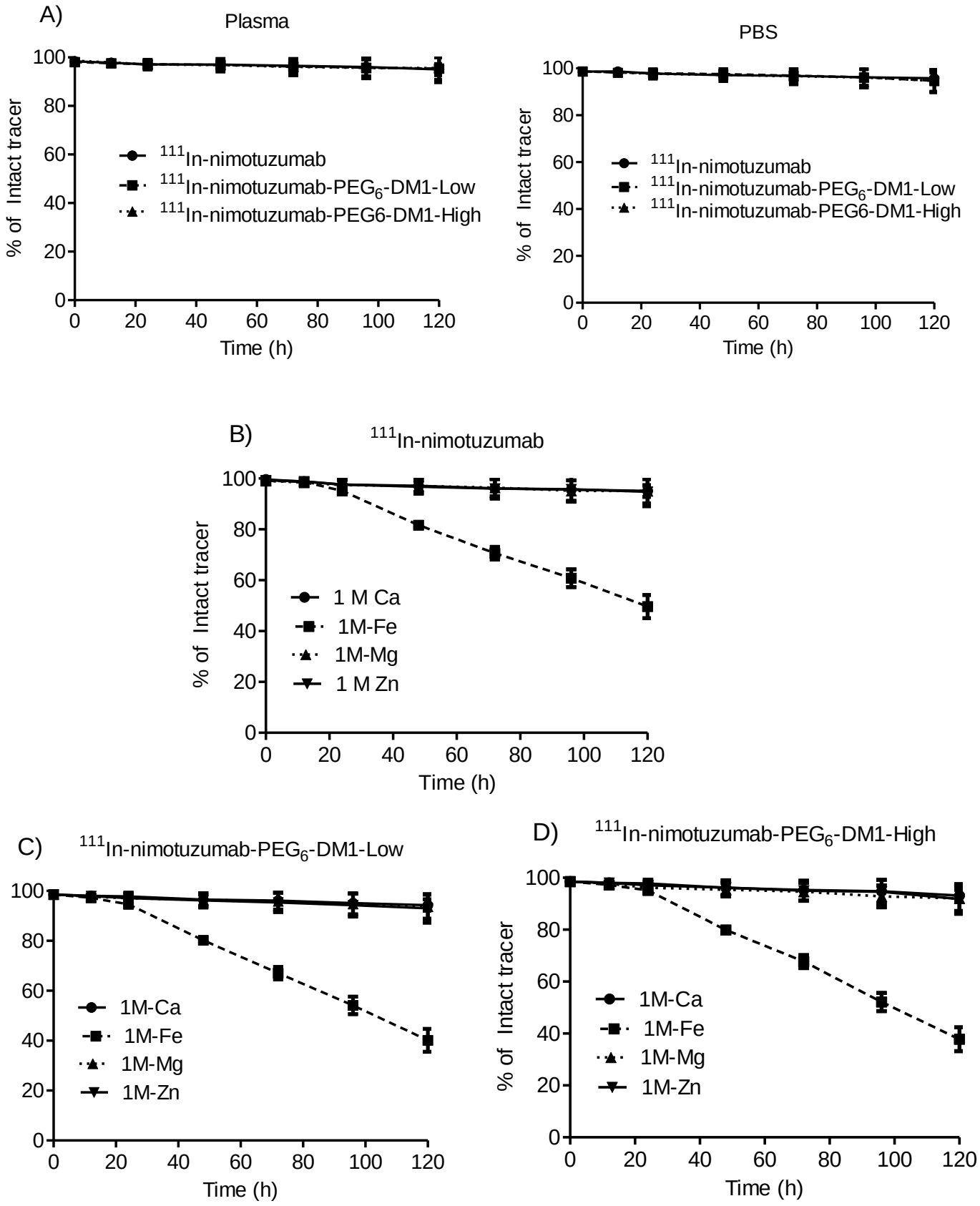
A


kDa
ladder
nimotuzumab
DOTA-nimotuzumab
DOTA-nimotuzumab-PEG6-DM1-Low
DOTA-nimotuzumab-PEG6-DM1-High
240
150
95
63
46
15
5
L
1
2
3
4

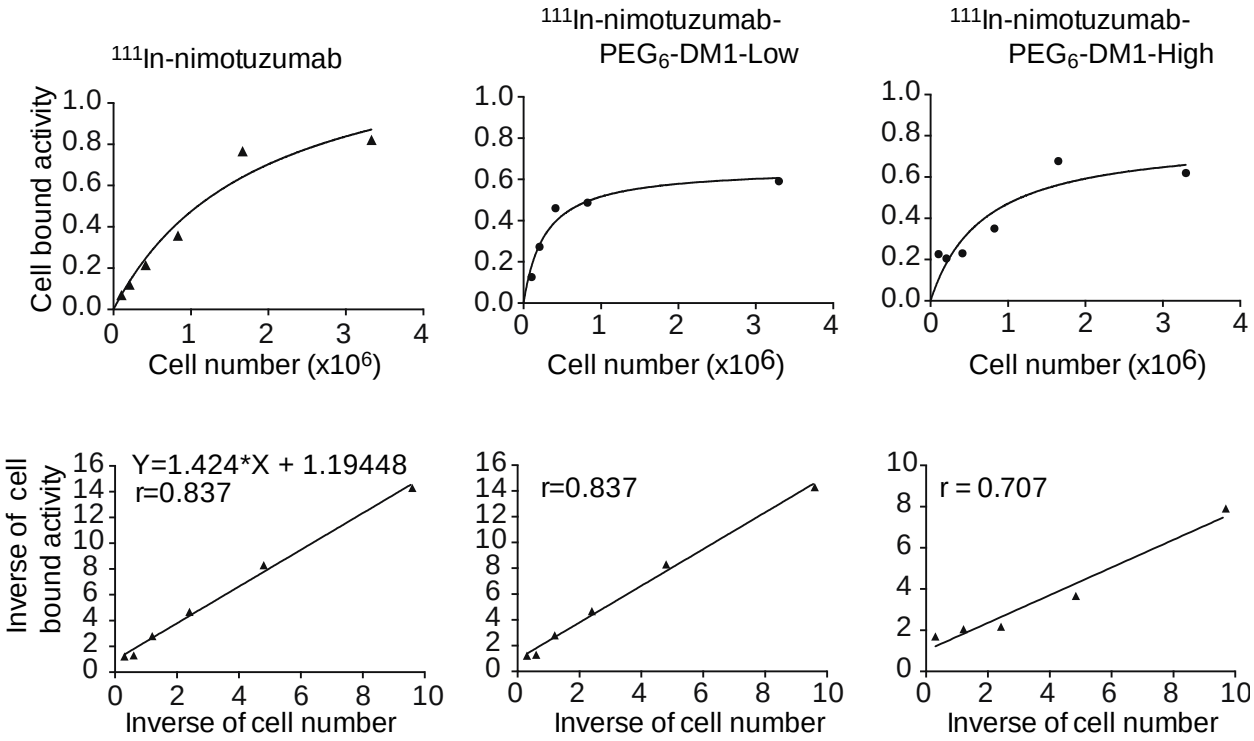
B



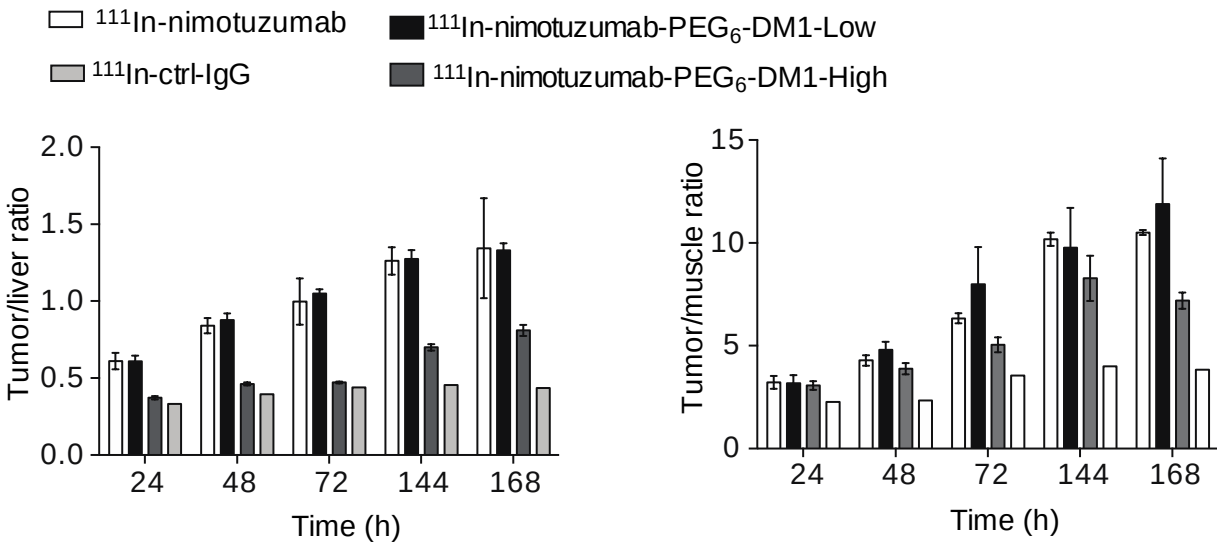
SUPPLEMENTAL FIGURE 5. Quality control of ADC using bioanalyzer (A) and biolayer interferometry (BLI) at different concentrations (55, 166 and 500 nM) of 9x-His tagged monomeric hEGFR, Leu 25 - Ser 645 (1001-H08H, Sino Biological) (B).


A)
Plasma
% of Intact tracer
Time (h)
111In-nimotuzumab
111In-nimotuzumab-PEG6-DM1-Low
111In-nimotuzumab-PEG6-DM1-High
PBS
B)
111In-nimotuzumab
1 M Ca
1M-Fe
1M-Mg
1 M Zn
C)
111In-nimotuzumab-PEG6-DM1-Low
1M-Ca
1M-Fe
1M-Mg
1M-Zn
D)
111In-nimotuzumab-PEG6-DM1-High

SUPPLEMENTAL FIGURE 6. A: *In vitro* stability of radioimmunoconjugates in plasma and PBS. B – D: iTLC analyses of the stability of ^{111}In-nimotuzumab, ^{111}In-nimotuzumab-PEG$_6$-DM1 and ^{111}In-nimotuzumab-PEG$_6$-DM1-High under conditions of large excess (1 M) of different metal ions *in vitro*.



SUPPLEMENTAL FIGURE 7. Immunoreactive fractions of ^{111}In-nimotuzumab, ^{111}In-nimotuzumab-PEG$_6$-DM1-Low and ^{111}In-nimotuzumab-PEG$_6$-DM1-High. On the linear regression plot the value r = immunoreactive fraction of the radioimmunoconjugate



SUPPLEMENTAL FIGURE 8 Tumor-to-liver (A) and tumor-to-muscle (B) ratios of the radioimmunocojugates. Values of percentage of injected activity per volume (%IA/cc) for the organs were used to generate the ratios. The ratios for ^{111}In-nimotuzumab and ^{111}In-nimotuzumab-PEG$_6$-DM1-Low increased over time, until the end of the study period (168 h p.i.). ^{111}In-nimotuzumab-PEG$_6$-DM1-High increased until 144 h p.i. when the Tumor/liver ratio began to decrease. ^{111}In-ctrl-IgG remain relatively constant throughout.