In situ identification of Gram-negative bacteria in human lungs using a topical fluorescent peptide targeting lipid A

Acknowledgements: We acknowledge technical assistance of flow cytometry from Shona Johnstone and chemical characterization support from Dr Marc Vendrell. This research was supported by the National Institute for Health Research (NIHR) BRC GMP Unit at Guy's and St Thomas' NHS Foundation Trust and NIHR Biomedical Research Centre based at Guy's and St Thomas' NHS Foundation Trust and King's College London. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health. **Funding:** We would like to thank the Wellcome Trust & Department of Health Healthcare Innovation Challenge Fund (HICF-0510-069) for supporting the staff involved in this study and the Proteus Engineering and Physical Sciences Research Council Interdisciplinary Research Collaboration (EP/K03197X/1).

A**uthor Contributions:** SVC, AMF, TA, NA and MB performed chemical design and synthesis. ARA, NM, ES, BM, CH, MB and KD designed and performed the in vitro biological assays. ES and KD designed and performed the murine work. ARA, NM, TC, DSC, ES and KD designed, set-up and performed the *ex vivo* ovine lung experiments. ARA, TC, AM, ATH, AMS, TW, CH and KD designed and undertook the *in vivo* study. ARA, NM and KD undertook data analysis. NH and JG provided cells from BALF and clinical isolates of bacterial strains and microbiology guidance respectively. CG designed the image analysis algorithm with input from ARA, NM and KD. CH, MB, TW and KD conceived and supervised the project. ARA and KD wrote the manuscript. All authors approved the manuscript. **Competing Interests:** CH, MB and KD are founder directors and have a shareholding in Edinburgh Molecular Imaging Ltd. KD has received travel and attendance fees from Mauna Kea Technologies as a consultant for an advisory board. ARA, TC, NM and KD have received travel fees for attendance at educational conferences supported by an unrestricted educational grant from Mauna Kea Technologies.

Figures 1-5 with legends below.

Supplemental information contained within a single file.

Movies 1-13 uploaded separately.

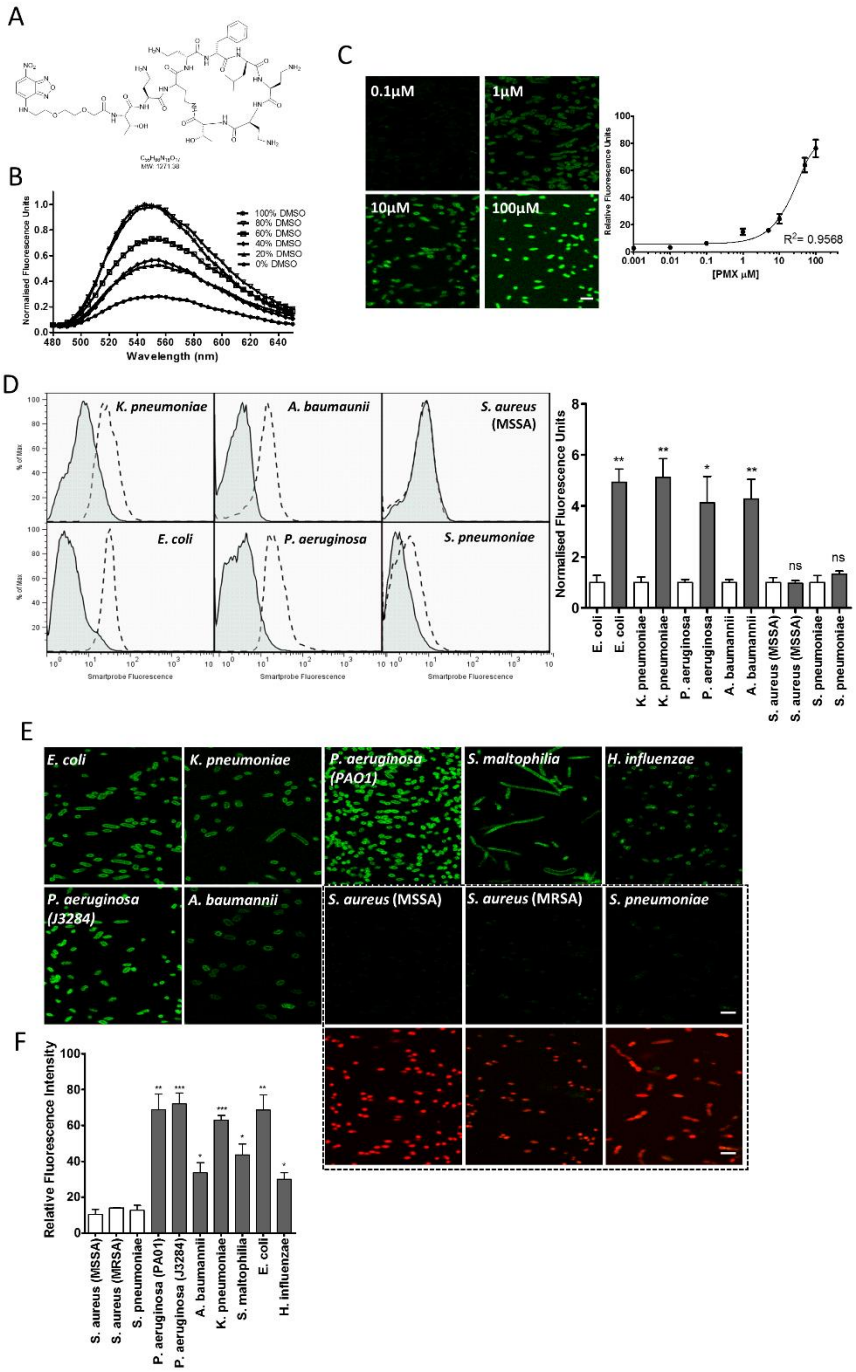

Figure 1: NBD-PMX labels gram-negative bacteria in a concentration dependent manner, with fluorescence amplification in hydrophobic environments. A) Structure of NBD-PMX; B) Fluorescence increase in increasing concentrations of Dimethyl sulfoxide (DMSO) (NBD-PMX at 5µM), n=3. C) Fluorescence quantification of *P. aeruginosa* imaged on a benchtop confocal microscope in the continued presence of increasing concentrations of NBD-PMX, demonstrating a concentration dependent fluorescent signal. Images show representative images at denoted concentration of NBD-PMX, scale bar represents 5µm. Each point on graph represents the mean (+/- SEM) of three independent experiments where at least three fields of view were quantified with a single site non-linear fit of data. D) Representative flow histograms for unstained bacteria (grey histograms) or NBD-PMX stained bacteria (5µM) (dotted line) demonstrating a greater fluorescence intensity increase for gram-negative than gram-positive bacteria. Graph shows quantification of flow cytometry data for unstained bacteria (white bars, normalized) and NBD-PMX (grey bars) demonstrating a significant increase in fluorescence for gram-negative bacteria, but no significant increase for gram positive bacteria. Bars represent means (+/- SEM) from three independent experiments, analysis is by students t-test, ns-not significant, *=p<0.05, **=p<0.01. E) Bacterial panel with NBD-PMX 1µM (green) and counterstain with Syto-82 (red). Gram-positive bacteria (bounded by box) display minimal/no labelling compared and are shown with their counterstain to demonstrate correct focal plane, scale bar represents 5µm. F) Quantification of bacterial panel with NBD-PMX (1µM) with gram-positive bacteria (white bars) and gram-negative (black bars), showing high intensity selective labelling of gram-negative bacteria compared with gram-positive. All gram-negatives showed a statistically significant increase over all gram-positives, bars show mean fluorescence (+/- SEM) from three independent experiments, where at least three fields of view were assessed. Analysis by Students t-test, *=p<0.05, **= p<0.01, ***=p<0.001.

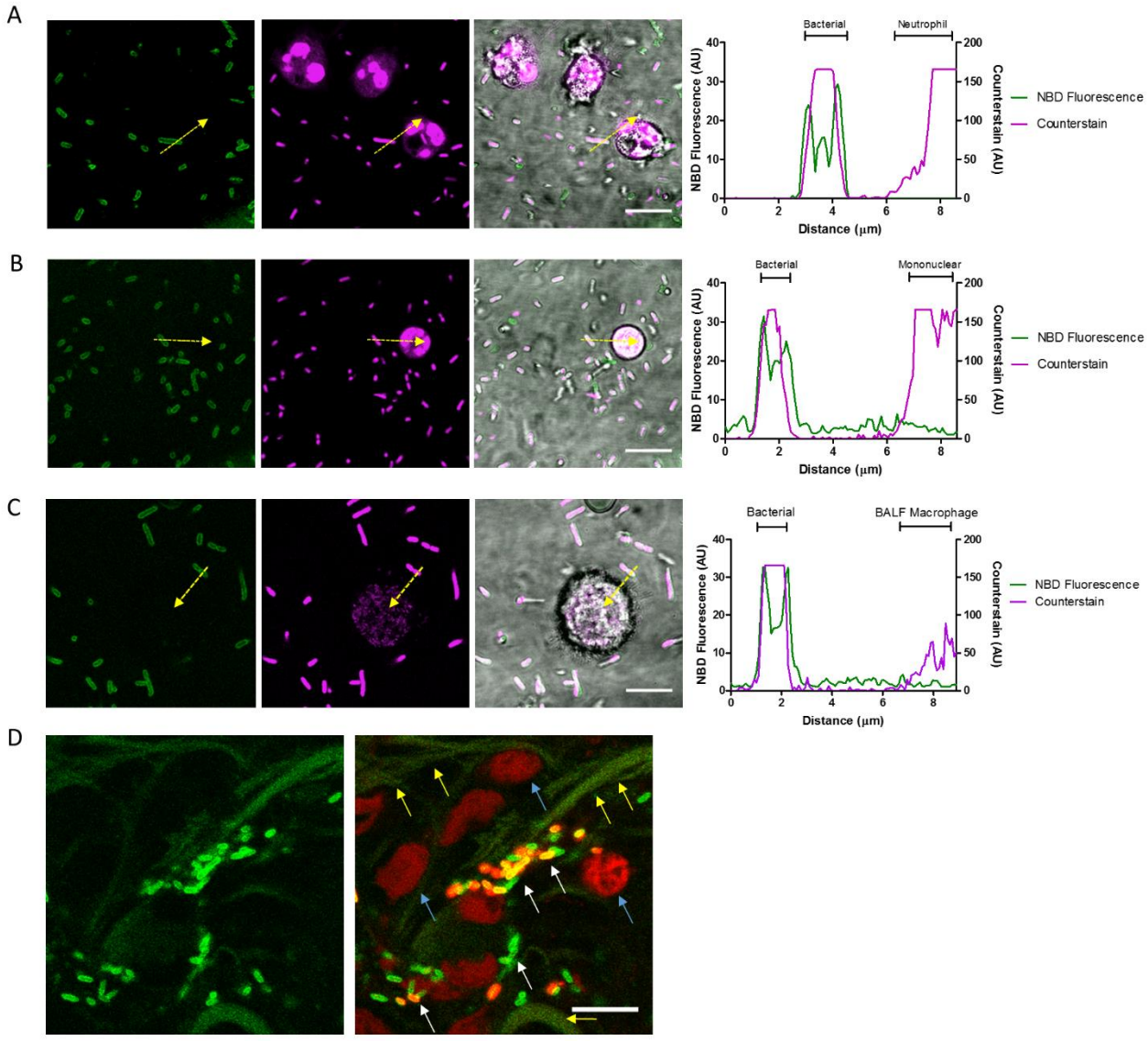

Figure 2: NBD-PMX demonstrated selectivity for bacteria over mammalian cells. Co-culture experiments of freshly isolated human neutrophils (A), freshly isolated human mononuclear cells (B) and human alveolar macrophages retrieved on bronchoalveolar lavage (C) with *P. aeruginosa* imaged in the continued presence of with NBD-PMX (5μM). Green panels demonstrate NBD fluorescence, middle panels demonstrate nuclear counterstain (Syto-60) and merge images shown on right. Plot profiles corresponding to yellow arrows shown on right with labelling of bacterial but not mammalian cell membranes. D) Confocal image of human lung tissue co-cultured with bacteria and imaged following labelling with NBD-PMX (5μM). 2 panels; left panel shows NBD and autofluorescence with excitation at 488nm and right panel shows merge with counterstain. White arrows indicate bacterial labelling, blue arrows demonstrate epithelial cells and yellow arrows demonstrate elastin autofluorescence. All experiments n=3, representative images shown, Scale bar represents 10μm.

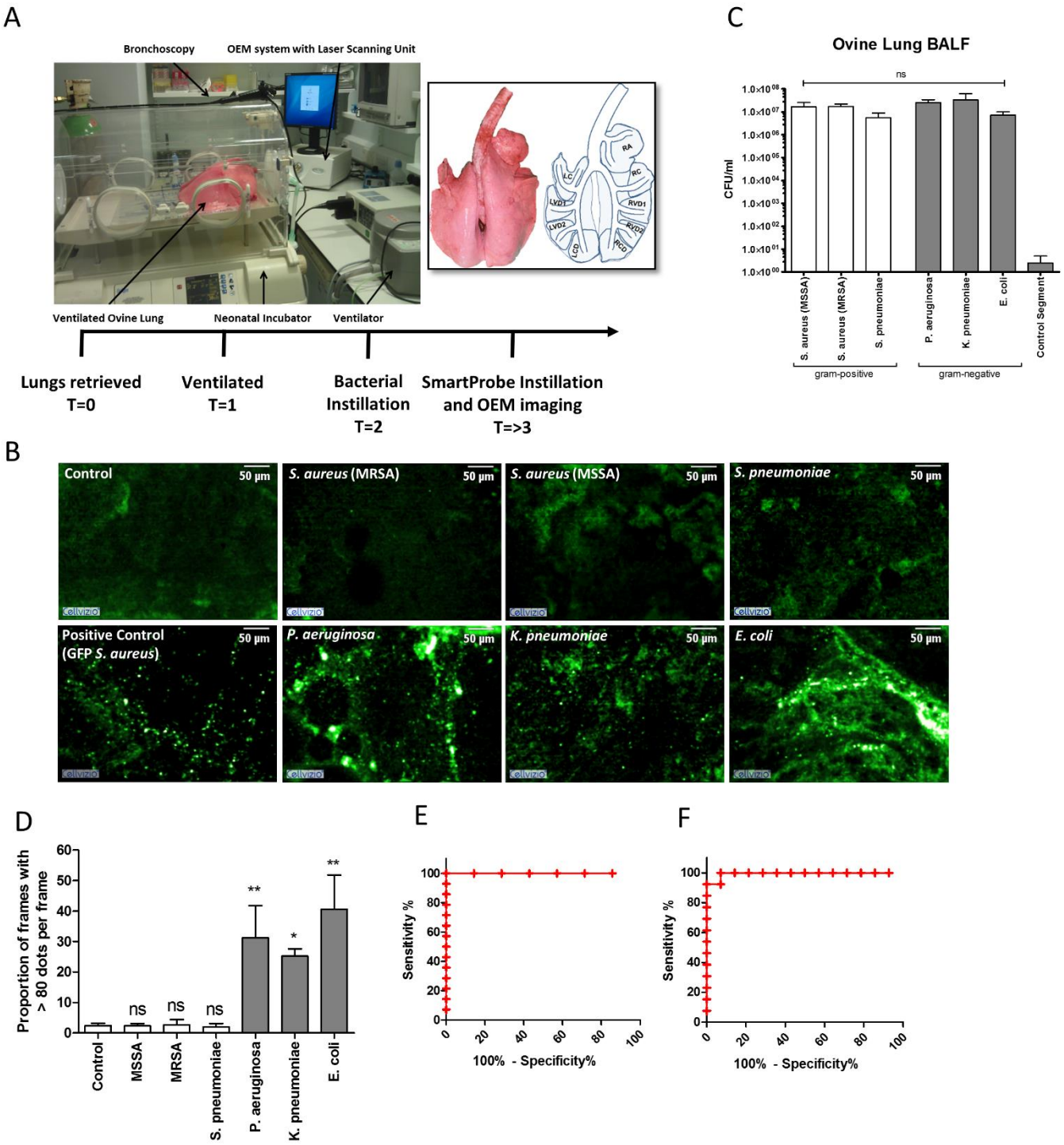

Figure 3: NBD-PMX labelled gram-negative, but not gram-positive bacteria *in situ* in *ex vivo* ovine lungs. A) Experimental set-up. Image demonstrating the anatomically distinct pulmonary segments of the ovine lung and timeline outlining the experimental protocol of retrieval, ventilation, bacterial instillation and NBD-PMX instillation and OEM imaging. B) Representative OEM images of no bacterial signal in the control or gram-positive bacteria segments (top panels), but a punctate bacterial fluorescent signal in the positive control (GFP *S. aureus*) and gram-negative segments. C) Lavage was enumerated for bacterial counts. Bacterial segments revealed no significant differences in bacterial counts, n=4 for all bacteria except *S. pneumoniae* where n=3, analysis by one-way ANOVA, ns=not significant. D) Analysis of entire videos for control segments (n=7), MSSA (n=5), MRSA (n=4), *S. pneumoniae* (n=4), *P. aeruginosa* (n=4), *K. pneumoniae* (n=5) and *E.coli* segments (n=5) demonstrating a significantly higher proportion of frames with >80 dots per frame for the gram-negative (grey bars) but not the gram-positive segments (white bars) when compared to control, bars represent mean (+/- SEM), *=p<0.05, **=p<0.01, ns=not significant, Students t-test. Receiver operator characteristics of image analysis videos; E) For control (n=7) or all gram-negative videos (n=14) the area under the curve was 1.0 (95% Confidence intervals (95%CI) 1.0-1.0), p=0.0002592. F) For gram-negative segments (n=14) compared to gram-positive segments (n=13) the AUC was 0.9945 (95%CI 0.9768-1.012), p<0.001.

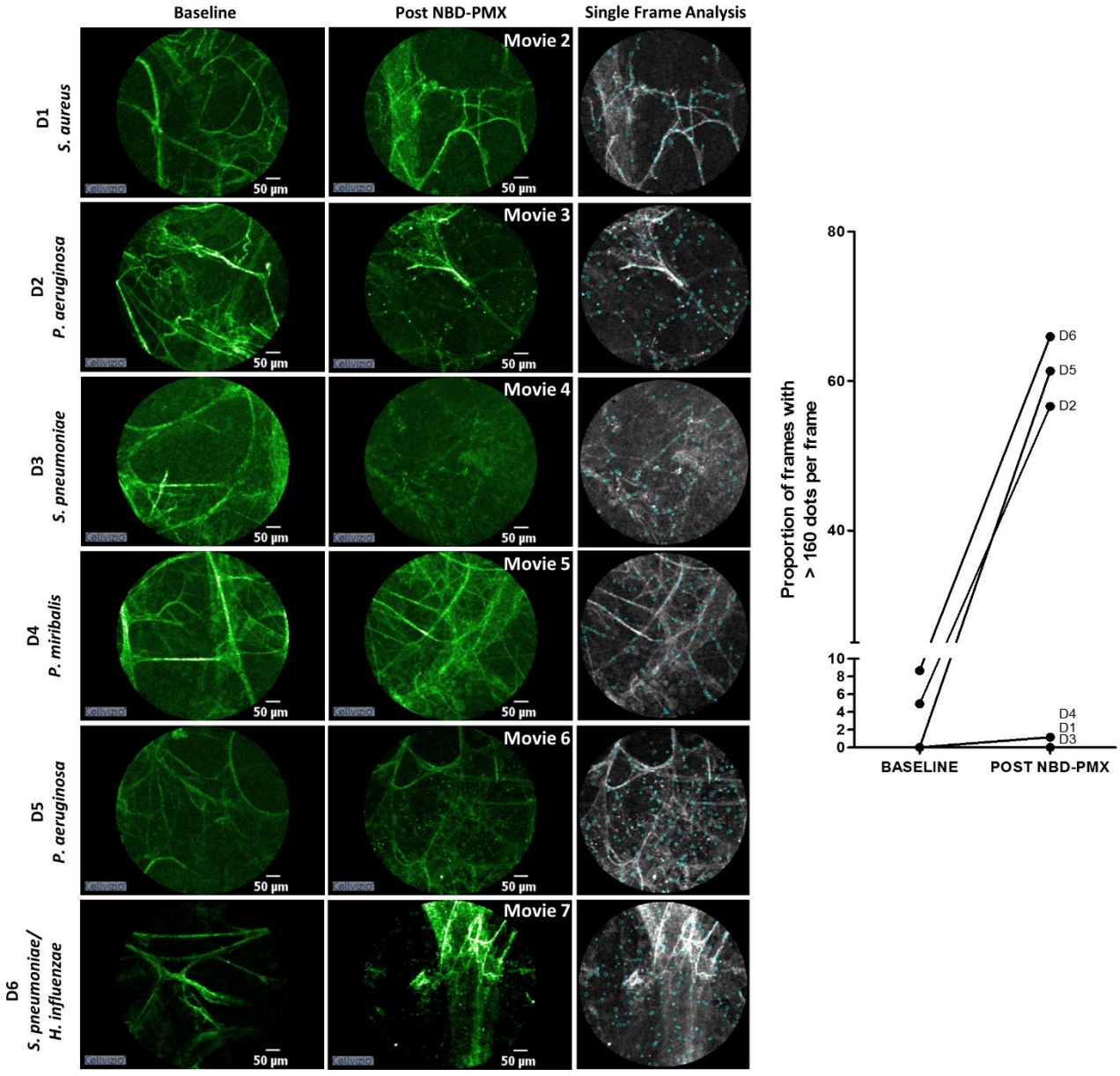

Figure 4: NBD-PMX labels gram-negative bacteria *in vivo* in humans when administered endobronchially and imaged with OEM. Representative alveolar images of baseline imaging (left) and post administration of NBD-PMX in six patients. D2, D5 and D6 demonstrated positive bacterial signal post NBD-PMX administration with dots detected in representative frame, defined by >10% frames with >160 punctate dots per frame. Graph demonstrates image analysis of individual frames from baseline imaging and post NBD-PMX demonstrating a higher proportion of frames with >160 dots per frame. Mean number of frames per video analysis was 443 frames, data shown as proportion of frames with >160 dots detected per frame.

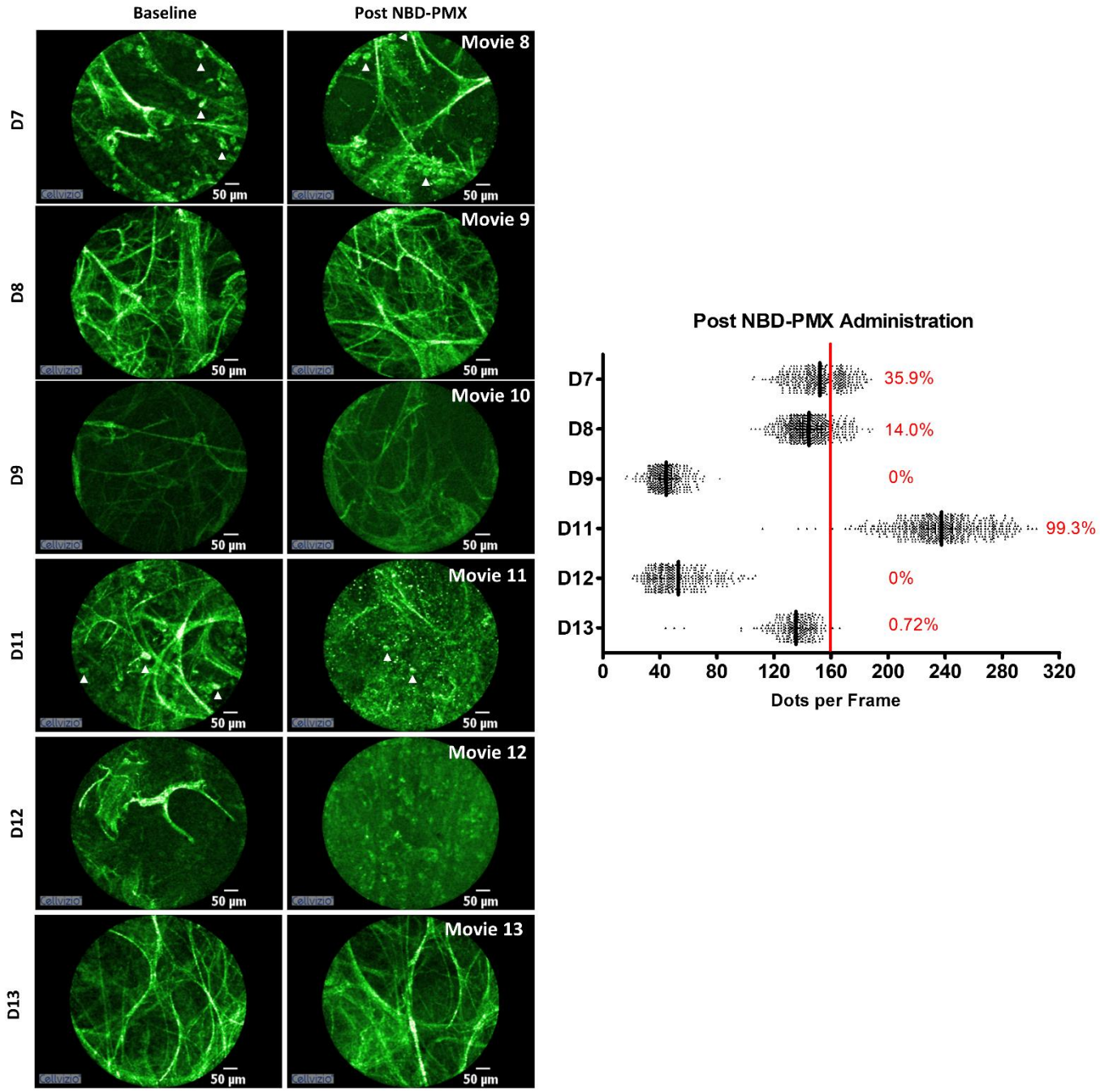

Figure 5: NBD-PMX administration and imaging in six ventilated patients in ICU. Representative alveolar images of baseline imaging (left) and post administration of NBD-PMX in six patients where pneumonia was suspected. D7 and D11 demonstrates cellular infiltrate (cells shown by white arrows) and presence of alveolar punctate signal consistent with bacterial presence. D8 and D13 demonstrates condensed elastin consistent with atelectasis, and both D9 and D12 demonstrate absence of alveolar bacterial signal. D12 demonstrates absence of alveolar structure consistent with the development of an abscess. Graph demonstrates the frame-by-frame analysis of each video sequence post NBD-PMX administration. Each point represents a single frame analysis and line represents the mean, with the % above the threshold of 160 dots per frame shown on right in red.

Immediate *In Situ* Identification of Gram-negative Bacteria in Human Lungs Using a Topical Fluorescent Peptide Targeted Against Lipid A

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Supplementary Information

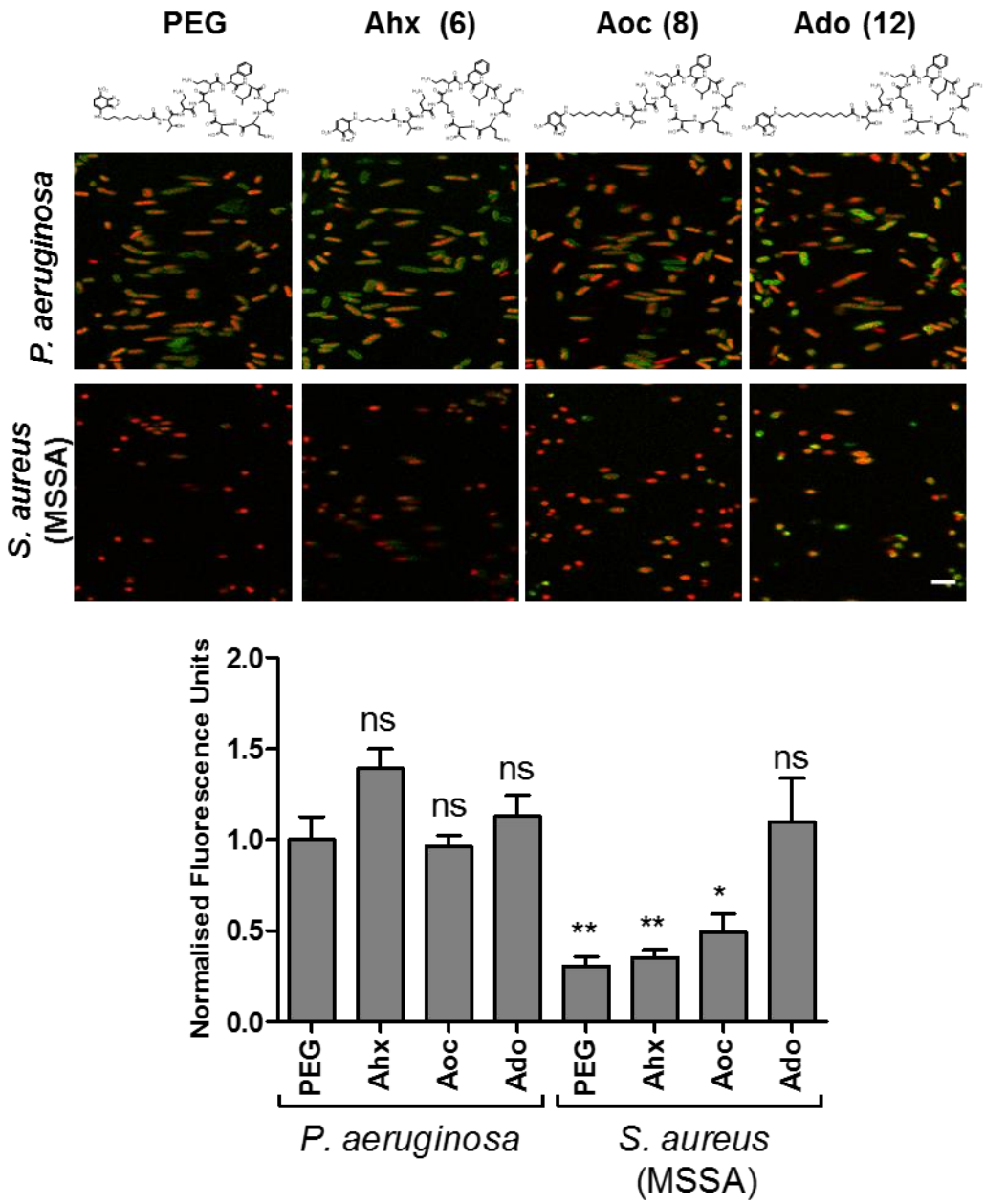


Figure S1: Progressive lengthening of the linker leads to loss of gram-selectivity. NBD-PMX constructs with Amino-3,6-dioxaoctanoic PEG2 (PEG), Aminohexanoic (Ahx) (6), Aminooctanoic (Aoc) (8) and Aminododecanoic (12) (all at 1µM) demonstrating similar fluorescence (green in panels) intensities for *P. aeruginosa* but increasing fluorescence for *Methicillin sensitive S. aureus (*MSSA) with representative merged images (with Syto-82 counterstain in red) (scale bar 5µm). Lower graph shows quantification of data and demonstrates no significant fluorescence for *P. aeruginosa* with the different constructs, but progressively increased fluorescence signal with MSSA. All data normalised and analyses compared to NBD-PMX with PEG on *P. aeruginosa*. Bars represent mean (+/- SEM), n=3, ns=not significant, *=p<0.05, **=p<0.01.

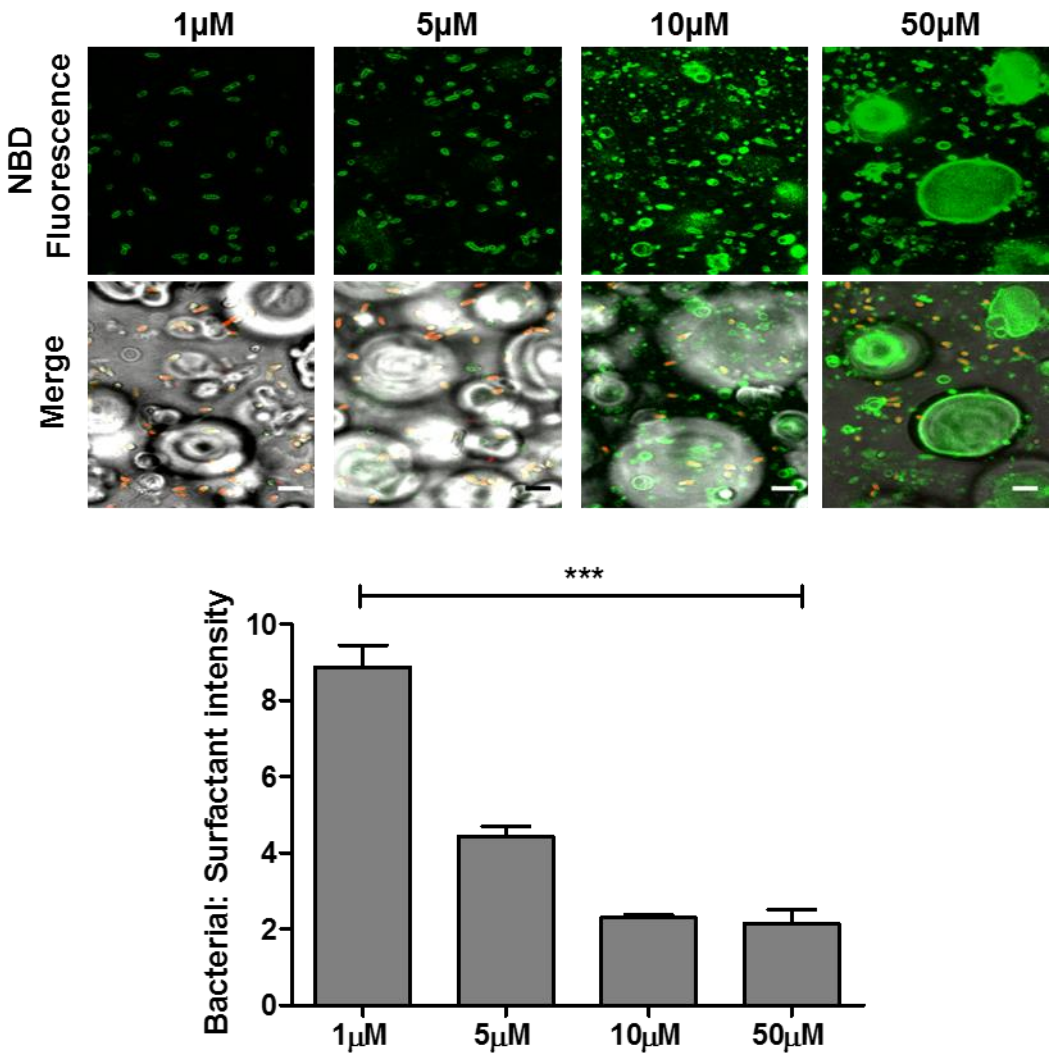


Figure S2: NBD-PMX has improved signal-to-noise at lower concentrations in the presence of pulmonary surfactant. NBD-PMX displayed increasing surfactant labelling at higher concentrations. Upper panels show fluorescence and brightfield merged images Lower graph displays quantification of bacterial fluorescence to surfactant fluorescence as a ratio. At all concentrations, the fluorescence of bacteria remain higher than surfactant. Bars represent mean (+/-SEM), n=3, analysis by one way ANOVA, ***=p<0.001, scale bar represents 5µm.

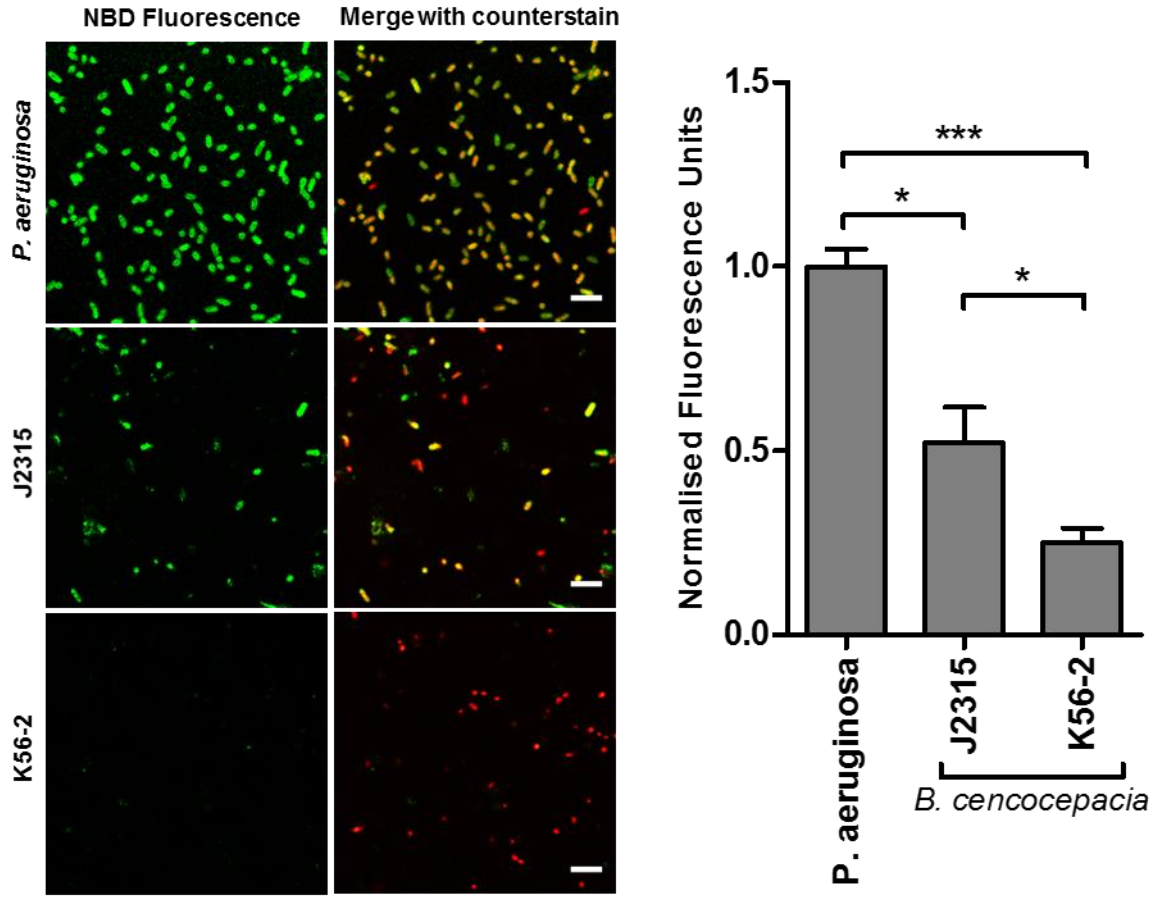


Figure S3: NBD-PMX binding with polymyxin resistant strains of *B. cenocepacia* is reduced compared to *P. aeruginosa*. Representative images (left) and quantification of fluorescence (right) of two forms of B. *cenocepacia*, with either depleted rough LPS (J2315) or smooth LPS but a mutated lipid A component (K56-2) LPS, and compared to *P. aeruginosa*. Data demonstrated there was a significant reduction in fluorescence with both strains compared to *P. aeruginosa* and a lower fluorescence intensity with K56-2 variant when compared to the J2315, bars represent mean (+/- SEM), n=4, *=p<0.05, ***=p<0.001, scale bar represents 5µm.

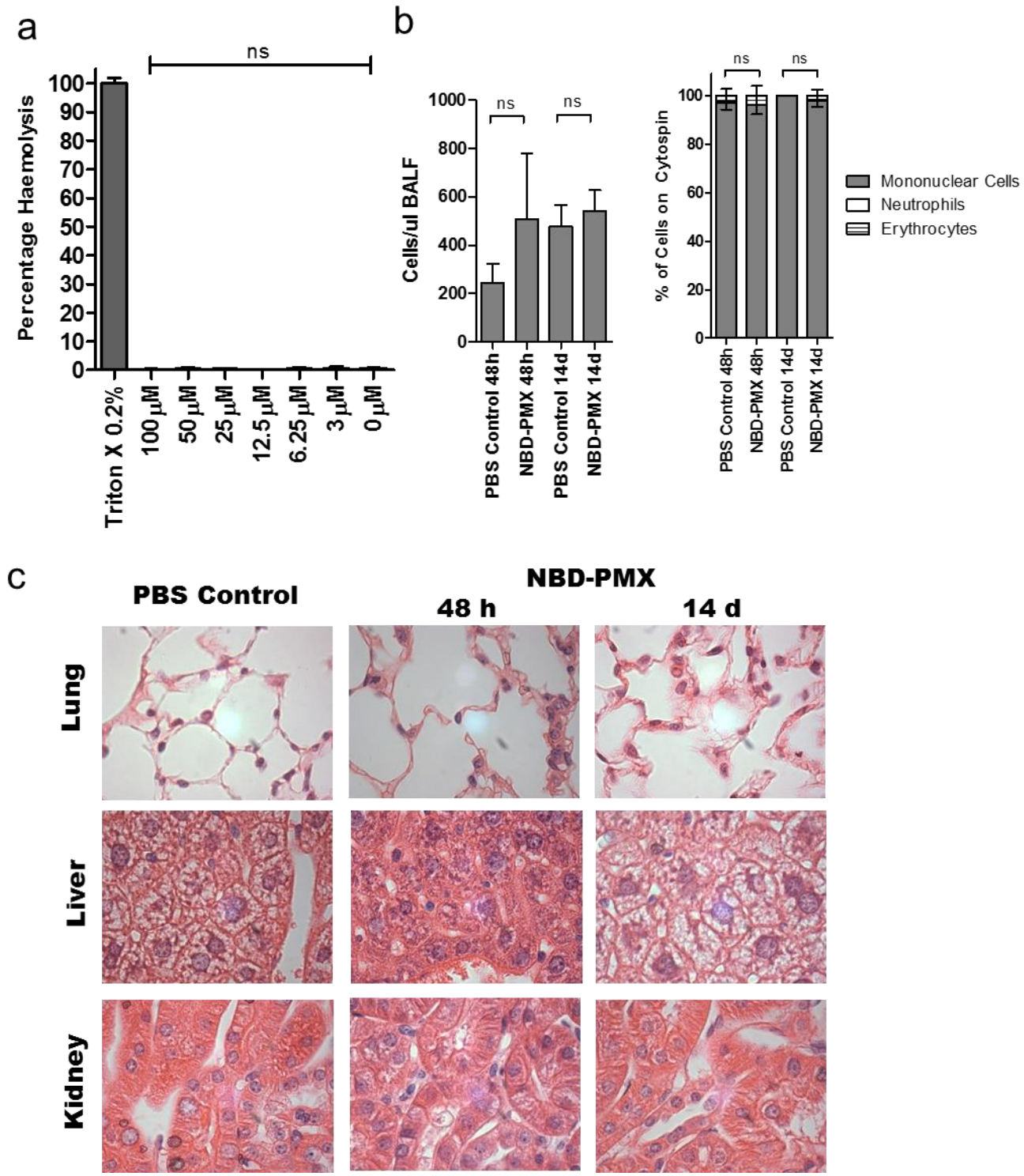


Figure S4: NBD-PMX demonstrated no cell membrane toxicity and no *in vivo* toxicity after intratracheal instillation in mice. A) No red blood cell haemolysis observed for NBD-PMX concentrations up to 100µM, n=3, bars represent mean (+/-SEM) of three independent procedures performed in duplicate. Positive control was 0.2% Triton-X and values corrected to represent 100% haemolysis for Triton-X. Statistical analysis by ANOVA., ns=not significant. B) BALF at 48 hours following intratracheal instillation of 100 micrograms of NBD-PMX (vs vehicle PBS control) demonstrated no increase in cells and no significant increase in neutrophil counts, bars represent mean (+/-SD), n=3. C) Representative histology of animals at 48 hours (x100) demonstrated no changes to microscopic architecture in lung, liver or kidney tissue.

	Control (Male)			NBD-PMX (Male)				Control (Female)			NBD-PMX (Female)			
Day (post dosing)	**Mean weight (g)**	**SD**	**n**	**Mean weight (g)**	**SD**	**n**		**Mean weight (g)**	**SD**	**n**	**Mean weight (g)**	**SD**	**n**	
1	140.2	9.71	14	140.4	10.93	15	ns	113.4	10.49	15	113.3	10.46	15	ns
2	144.8	9.5	15	146	13.96	15	ns	116.4	9.81	15	115.9	11.14	15	ns
3	152.8	13.68	5	163	11.69	5	ns	128.2	8.51	5	123.3	14.67	5	ns
4	157	12.94	5	165.2	12.51	5	ns	128.4	6.55	5	124.4	12.49	5	ns
8	179.3	14.33	5	187.9	12.61	5	ns	139.4	5.04	5	138.3	13.94	5	ns
11	194.5	12.48	5	206.2	12.18	5	ns	148.1	4.67	5	146.7	15.8	5	ns
14	210.8	10.86	5	222.2	13.79	5	ns	156.1	2.28	5	154.3	16.76	5	ns

		Control (Male)			NBD-PMX (Male)				Control (Female)			NBD-PMX (Female)			
		Mean	**SD**	**n**	**Mean**	**SD**	**n**		**Mean**	**SD**	**n**	**Mean**	**SD**	**n**	
Hb (g/dL)	Day 3	13.1	0.47	10	13.2	0.46	6	ns	13.7	0.25	4	13.6	0.45	9	ns
	Day 15	14	0.24	4	14.1	0.45	5	ns	14.2	0.78	5	14.5	0.71	5	ns
WCC (10^9/L)	Day 3	3.4	0.87	10	3.3	1.18	6	ns	2.9	0.79	4	2.7	0.6	9	ns
	Day 15	5.7	1.42	4	4.5	1.9	5	ns	3.7	1.27	5	5.2	0.55	5	*
Plt (10^9/L)	Day 3	1037	94.7	10	907	102.3	6	*	1009	113.7	4	1063	59.4	9	ns
	Day 15	953	77	4	958	53.1	5	ns	1000	123.1	5	1024	78.3	5	ns
PT (s)	Day 3	22.5	0.69	8	22.4	0.7	8	ns	21.7	1.16	4	21.6	1.3	10	ns
	Day 15	22.6	0.73	5	22.3	0.52	5	ns	24.5	0.79	5	24.7	0.98	5	ns
Fibrinogen (g/L)	Day 3	1.58	0.076	8	1.56	0.086	8	ns	1.52	0.057	4	1.56	0.052	10	ns
	Day 15	1.59	0.063	5	1.64	0.073	5	ns	1.4	0.076	5	1.39	0.09	5	ns
Urea (mmol/L)	Day 3	5.6	1.01	10	5.8	0.42	10	ns	4.8	0.83	10	6	1.06	10	**
	Day 15	7.2	1.15	5	7.2	1.54	5	ns	6.4	0.68	5	5.9	1.08	5	ns
Hcre (µmol/L)	Day 3	17	1.1	10	17	1.6	10	ns	16	1.3	10	17	1.0	10	*
	Day 15	21	1.5	5	21	2.8	5	ns	21	1.1	5	20	1.2	5	ns
Na (mmol/L)	Day 3	140	1.3	10	140	1.4	10	ns	140	1.4	10	140	1.8	10	ns
	Day 15	140	1.6	5	140	0.8	5	ns	140	1.1	5	140	1.6	5	ns
K (mmol/L)	Day 3	3.6	0.11	10	4	0.45	10	**	3.7	0.21	10	3.6	0.26	10	ns

	Day 15	3.9	0.16	5	3.6	0.18	5	*	3.5	0.22	5	3.5	0.13	5	ns
AST (IU/L)	Day 3	73	11.5	10	75	11.9	10	ns	71	10.8	10	71	8.5	10	ns
	Day 15	49	6.2	5	56	8.4	5	ns	76	25	5	65	5.1	5	ns
ALT (IU/L)	Day 3	61	10.5	10	69	7.5	10	ns	47	8.9	10	53	12.7	10	ns
	Day 15	38	7.1	5	41	8.7	5	ns	35	8.7	5	31	9.8	5	ns
Glucose (mmol/L)	Day 3	6.4	0.66	10	6.7	1.47	10	ns	5.5	0.84	10	6	1.41	10	ns
	Day 15	9.5	2.42	5	7	0.58	5	**	5.5	0.99	5	5.8	0.55	5	ns
Total Protein (g/L)	Day 3	53	1.9	10	54	1.3	10	ns	54	3	10	56	1.7	10	ns
	Day 15	57	1.4	5	59	2.4	5	ns	59	3.4	5	58	1.8	5	ns
Albumin (g/L)	Day 3	36	2.2	10	38	1.8	10	*	39	2.7	10	39	2.4	10	ns
	Day 15	40	2.3	5	42	1.9	5	ns	42	3.5	5	42	2.1	5	ns
Cholesterol (mmol/L)	Day 3	2.9	0.39	10	2.7	0.36	10	ns	2.9	0.58	10	3.1	0.61	10	ns
	Day 15	2.3	0.37	5	2.5	0.81	5	ns	1.8	0.41	5	1.9	0.29	5	ns

Hb: Haemoglobin, WCC: total white cell count, Na: Sodium, K: Potassium, Plt: Platelet count, PT: Prothombin Time, ALT: Alanine Aminotransferase, AST: Aspartate Aminotransferase, Hcre: Enzymatic Creatinine.

Table S1: No significant changes in weight, haematological, coagulation or clinical chemistry parameters. Analysis by two-sample t-test; ns=not significant, *=p<0.05, **=P<0.01. Where significances were found the data was within the historical control ranges these changes were considered not to be of toxicological significance.

Conditions / Timepoint	Freezer -20 °C			
	RT / min	Conc. / µg per 5mL (Δ%)	Total imp. RRT(%)	pH
0	6.538 -	60.51 -	ND ND	7.5 -
48h	6.539 6.539	56.56 (6.5%) 56.18 (7.2%)	ND ND	7.5 7.5
1m	6.548 6.549	55.0 (9.1%) 53.44 (11.6%)	ND ND	7.5 7.5
3m	6.515 6.517	57.23 (5.4%) 49.44 (18%)	ND ND	7.5 7.5
5m	6.517 6.519	65.01 (7.4%) 61.58 (1.8%)	ND ND	7.5 7.5
6m	6.538 6.537	62.17 (2.7%) 61.59 (1.8%)	ND ND	7.5 7.5
10m	6.514 6.508	57.85 (4.4%) 55.43 (8.4%)	ND ND	7.5 7.5
18m	6.519 6.520	65.66 (+8.5%) 65.90 (+8.9%)	ND ND	7.5 7.5
24m	6.456 6.490	60.53(+0.03%) 57.00 (-5.8%)	ND ND	7.5 7.5

Table S2: NBD-PMX is stable in aqueous formulation over 24 months. Summary table of stability as assess by HPLC over 10 months stored under GMP frozen conditions (-20^{0}C). Data shown in blue is representing "inverted" position vials; Δ% is relative to time zero. Acceptance criteria for the GMP product**:** Retention time (RT) for NBD-PMX as 6.50 min (Range 6.30-6.75 min). HPLC assay concentration for technical batch is as 60.51µg/5mL (+/-25%). Total impurities – report as RRT (%) for each impurity. pH 7.5 (7.0-8.0).

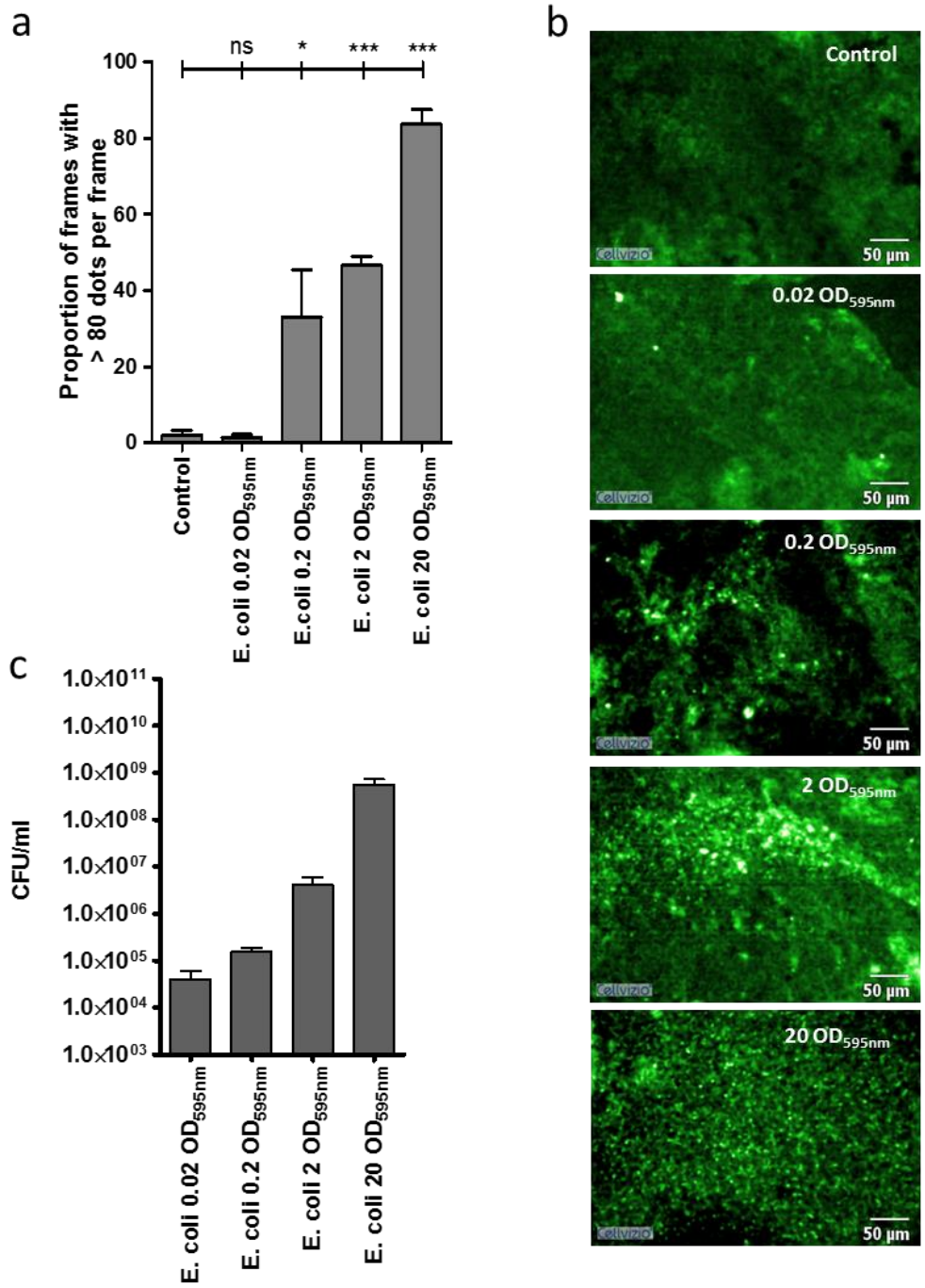


Figure S5: OEM coupled with NBD-PMX enables bioburden evaluation in the distal ovine lung. a) Proportion of positive frames for *E. coli* presence with increasing concentrations of bacteria with NBD-PMX instilled topically and imaged. n=3 for all conditions, bars represent mean (+/-SEM), analysis by students-t test, ns=not significant, *=p<0.05, ***=p<0.001, all analyses when compared to control segment. b) Representative images for each bacterial concentration and c) Bacterial yields from bacteria retrieved on lavage, confirming the increasing concentration of bacteria per pulmonary segment, n=3 for each concentration.

Patient	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**
Gender	F	F	F	M	F	M
Age	62	58	66	68	68	68
FEV1 (% predicted)	2.46 (98%)	1.54 (81%)	1.35 (66%)	1.32 (39%)	1.54 (76%)	2.75 (94%)
Bronchiectasis Location (CT scan) and Type	Tubular bronchiectasis, all lobes	Varicose bronchiectasis LUL and LLL. Tubular in other lobes	Tubular bronchiectasis in all lobes	Tubular bronchiectasis in all lobes	Tubular bronchiectasis in RLL and RML	Tubular bronchiectasis in RML and Lingula
OEM Imaging and NBD-PMX Administration	RML	LLL	RLL	RML	RML	RML
Hb (g/L) Pre-Bronchoscopy	-*	131	139	144	107	140
Hb (g/L) Post-Bronchoscopy	151	129	141	138	104	139
WCC (10^9/L) (109/L) Pre-Bronchoscopy	-*	5.9	338	8.5	5	9.6
WCC (10^9/L) Post-Bronchoscopy	15.8	9.9	350	13.3	8.4	11
Platelets (10^9/L) Pre-Bronchoscopy	-*	394	313	295	390	234
Platelets (10^9/L) Post-Bronchoscopy	246	410	314	305	381	222
Sodium (mmol/L) Pre-Bronchoscopy	137	137	144	141	141	140
Sodium (mmol/L) Post-Bronchoscopy	139	139	141	142	139	135
Potassium (mmol/L) Pre-Bronchoscopy	4.4	4.8	4	4	4.2	3.3
Potassium (mmol/L) Post-Bronchoscopy	4	4.3	3.9	3.6	3.8	3.4
Urea (mmol/L) Pre-Bronchoscopy	3.8	6.4	5.9	6	5.8	9.9
Urea (mmol/L) Post-Bronchoscopy	3.4	6.1	6.1	6.4	5.8	10.6
Creatinine (µmol/L) Pre-Bronchoscopy	60	66	72	92	63	110
Creatinine (µmol/L) Post-Bronchoscopy	59	74	68	89	59	124
Bilirubin (µmol/L) Pre-Bronchoscopy	7	7	7	10	7	23
Bilirubin (µmol/L) Post-Bronchoscopy	8	7	7	8	6	22
ALT (U/L) Pre-Bronchoscopy	19	15	22	33	16	38
ALT (U/L) Post-Bronchoscopy	17	15	24	30	15	41
C-reactive Protein (mg/L) Pre-Bronchoscopy	<1	3	5	2	<1	30
C-reactive Protein (mg/L) Post-Bronchoscopy	<1	3	5	2	<1	30
	*= Insufficient Sample, Hb= Haemoglobin, WCC= White cell count, ALT= Alkaline Transferase LUL- Left upper lobe, LLL- Left lower lobe, RLL- Right lower lobe, RML- Right middle lobe					
Total Procedure Time (mins)	33	22	25	18	25	20
NBD-PMX Administration to End Procedure Including BAL (mins)	22	11	13	12	7	7
Total Number of Passes with OEM	4	3	3	5	4	5
Serious Adverse Events	None	None	None	None	None	None
Adverse Events	None	Mild Sore Throat	None	None	Mild Fever & New Infiltrate on chest radiograph (resolved)	Increased Sputum production
BAL/Sputum Growth	*S. aureus*	*P. aeruginosa*	*S. pneumoniae*	*P. miribalis*	*P. aeruginosa*	*S. pneumoniae/H. influenzae*
CFU/ml	2.8×10^5	1.7×10^5	8×10^5	2.7×10^6	2.1×10^4	$2.2 \times 10^5/3 \times 10^4$

Table S3: Bronchiectasis patient demographics, blood results, adverse events and microbiological growth.

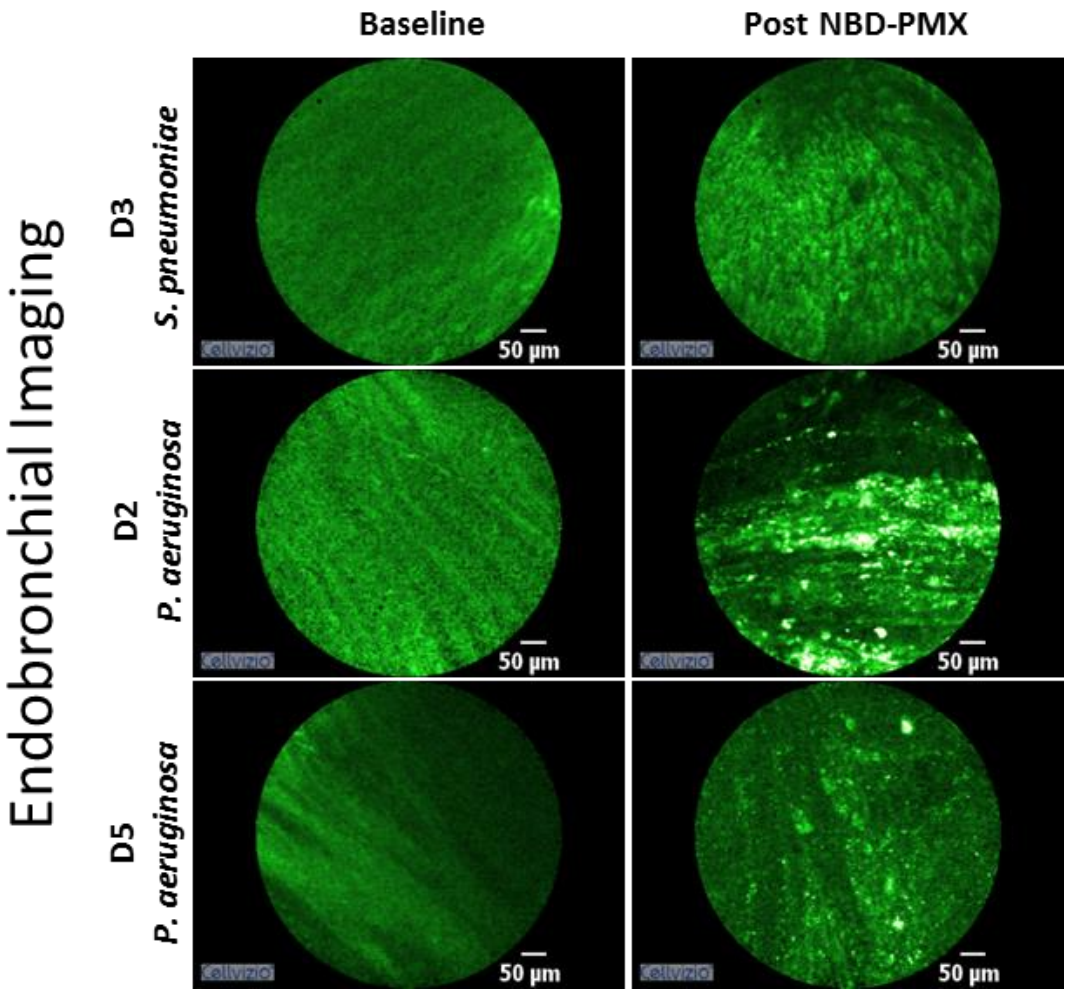


Figure S6: NBD-PMX labels gram-negative bacteria endobronchially. Representative images of bronchial structures demonstrating baseline (left) and following NBD-PMX administration *in vivo*. Bacterial presence, often in clumps, can be seen in patients with gram-negative bacteria (D2 and D5) but not seen in the patient with gram-positive bacteria (D3).

Patient	**D7**	**D8**	**D9**	**D11**	**D12**	**D13**
Gender	M	F	F	M	M	M
Age	72	72	71			
Primary diagnosis necessitating ICU admission	Post-operative care following oesophageal carcinoma surgery	Post-operative care following perforated gastric ulcer, re-admission with respiratory failure	Poly-trauma, pedestrian in road traffic accident	Respiratory failure secondary to pneumonia	Respiratory failure secondary to pneumonia	Respiratory failure, fluid overload and suspected pneumonia
Length of Time in ICU (days from admission to procedure)	9	3*	6	1	10	3
APACHE II Score (0-71)	12	24	14	21	24	31
Hospital Mortality Probability Score (%)	11.8	65.7	7.2	38.9	32.5	73.3
Imaging changes (CT scan or chest radiograph)	CT: RUL and RLL	Chest radiograph: RLL and LLL	Chest radiograph: LLL	Chest radiograph: RLL	CT: RML and RLL changes. Cavitation RML	Chest radiograph: RLL, RML, LLL and L effusion
OEM imaging and NBD-PMX administration	RLL	LLL	LLL	RLL	RLL	RML
Total procedure time (mins)	14	13	19	16	14	12
NBD-PMX administration to end procedure including BAL (mins)	4	7	10	11	12	8
Total number of passes with OEM	4	4	4	8	5	4
Adverse Events	Fever (unrelated)	None	None	None	Haemoptysis (minor)	None
Serious Adverse Events	Laparotomy for leaking jejunostomy (unrelated)	None	None	None	None	None
	*Re-admission to ICU following initial 23 day ICU stay LUL- Left upper lobe, LLL- Left lower lobe, RUL- Right upper lobe					
Hb (g/L) Pre-Bronchoscopy	101	77	85	145	125	81
Hb (g/L) Post-Bronchoscopy	97	77	81	132	114	86
WCC (10^9/L) (109/L) Pre-Bronchoscopy	23.4	14.6	8.4	11.9	11.5	5.4
WCC (10^9/L) Post-Bronchoscopy	25.8	13.9	8.6	14.1	11.4	7
Platelets (10^9/L) Pre-Bronchoscopy	409	496	146	278	209	115
Platelets (10^9/L) Post-Bronchoscopy	398	494	186	259	201	137
Sodium (mmol/L) Pre-Bronchoscopy	137	138	149	145	138	136
Sodium (mmol/L) Post-Bronchoscopy	135	136	148	144	137	136
Potassium (mmol/L) Pre-Bronchoscopy	4.4	4.6	4.0	3.2	4.2	4.3
Potassium (mmol/L) Post-Bronchoscopy	4.3	4.9	4.1	3.6	3.4	4.6

Urea (mmol/L) Pre-Bronchoscopy	4.1	10.6	7.1	5.7	8.3	10.3
Urea (mmol/L) Post-Bronchoscopy	4.0	10.5	7.9	6.2	8.7	9.2
Creatinine (µmol/L) Pre-Bronchoscopy	52	87	59	57	47	138
Creatinine (µmol/L) Post-Bronchoscopy	53	85	58	55	50	121
Bilirubin (µmol/L) Pre-Bronchoscopy	14	7	14	11	38	8
Bilirubin (µmol/L) Post-Bronchoscopy	15	6	14	9	30	7
ALT (U/L) Pre-Bronchoscopy	17	17	86	49	42	18
ALT (U/L) Post-Bronchoscopy	18	19	169	44	36	17
C-reactive Protein (mg/L) Pre-Bronchoscopy	416	173	81	ND	27	62
C-reactive Protein (mg/L) Post-Bronchoscopy	416	164	90	53	24	ND
	Hb= Haemoglobin, WCC= White cell count, ALT= Alkaline Transferase, ND= not done					
BAL Growth (Bacterial)	No growth	*S. maltophilia*	*P. aeruginosa*	*K. oxytoca*	No growth	Coagulase-negative *S. epidermidis* (respiratory tract commensal)
CFU/ml	-	1×10^4	1×10^2 (under VAP diagnostic threshold)	2×10^2	-	7×10^2
Animicrobial therapy prior to procedure	Metronidazole, Ciprofloxacin, Vancomycin and Fluconazole (anti-fungal)	None	None	Temocillin	Flucloxacillin and amoxicillin/clavulanic acid	Ceftriaxone and clarithromycin
Growth of other organisms from BAL	-	-	-	Candida albicans (moderate numbers)	Candida albicans (small numbers)	-
OEM Diagnosis	Pneumonia (VAP)	Atelectasis, No VAP	No VAP	Pneumonia	Pulmonary Abscess	Atelectasis, No VAP

Table S4: ICU patient demographics for six patients included in the analysis, blood results, adverse events and microbiological growth.

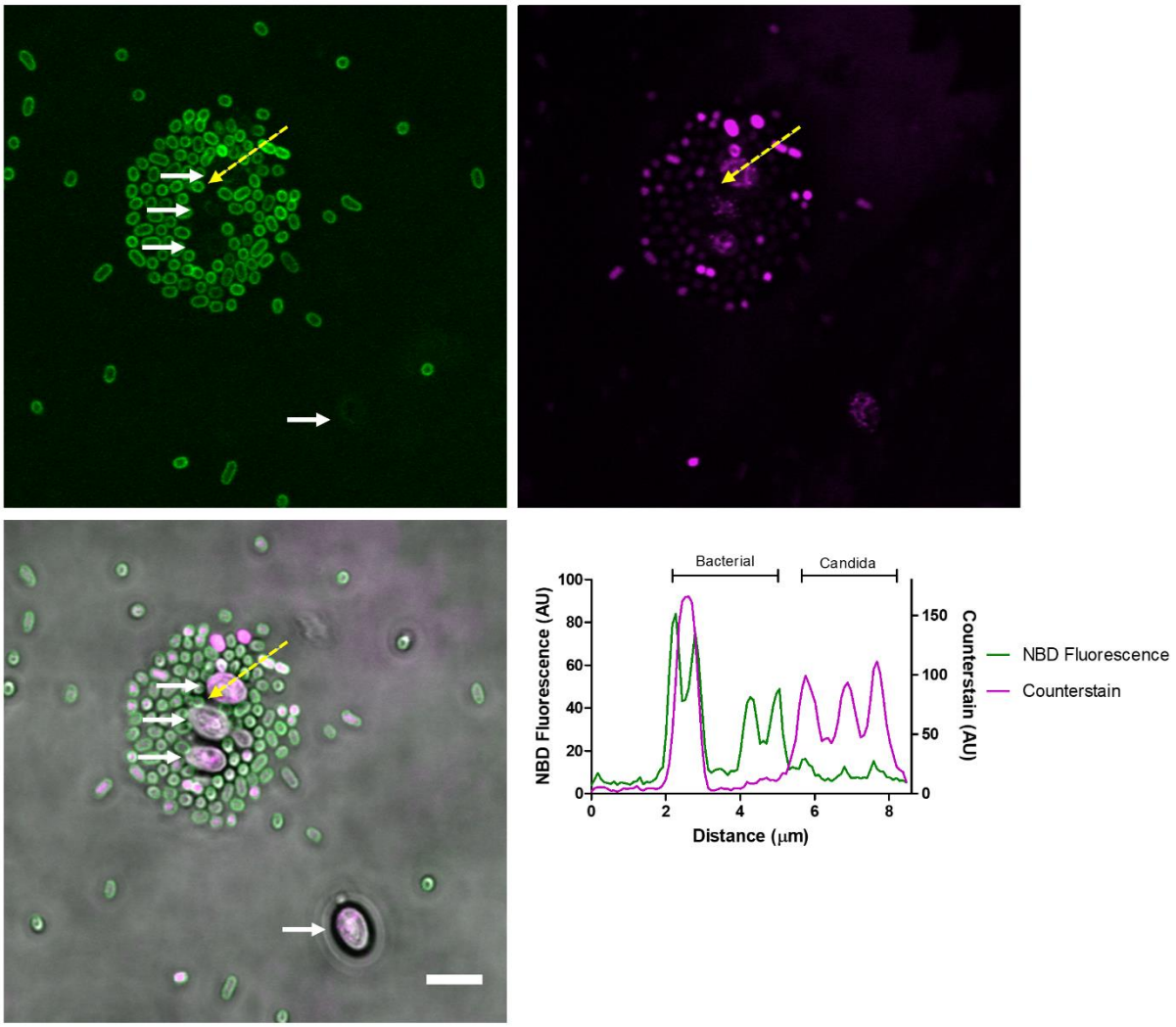


Figure S7: Ex-vivo confocal labelling of species from BAL in patient D11. Confocal images of bacteria and fungi isolated from BALF of patient D11 (identified as *K. oxytoca* and *C. albicans*) in co-culture in the presence of NBD-PMX (10µM). Upper left image shows NBD-PMX fluorescence of *K. oxytoca* labeling but none of *C. albicans* (white arrows). Upper tight image shows counterstain and lower left panel demonstrates merge, scale bar 5 µm. Plot profile along yellow dashed line demonstrates labeling with counterstain but not NBD-PMX of Candida.

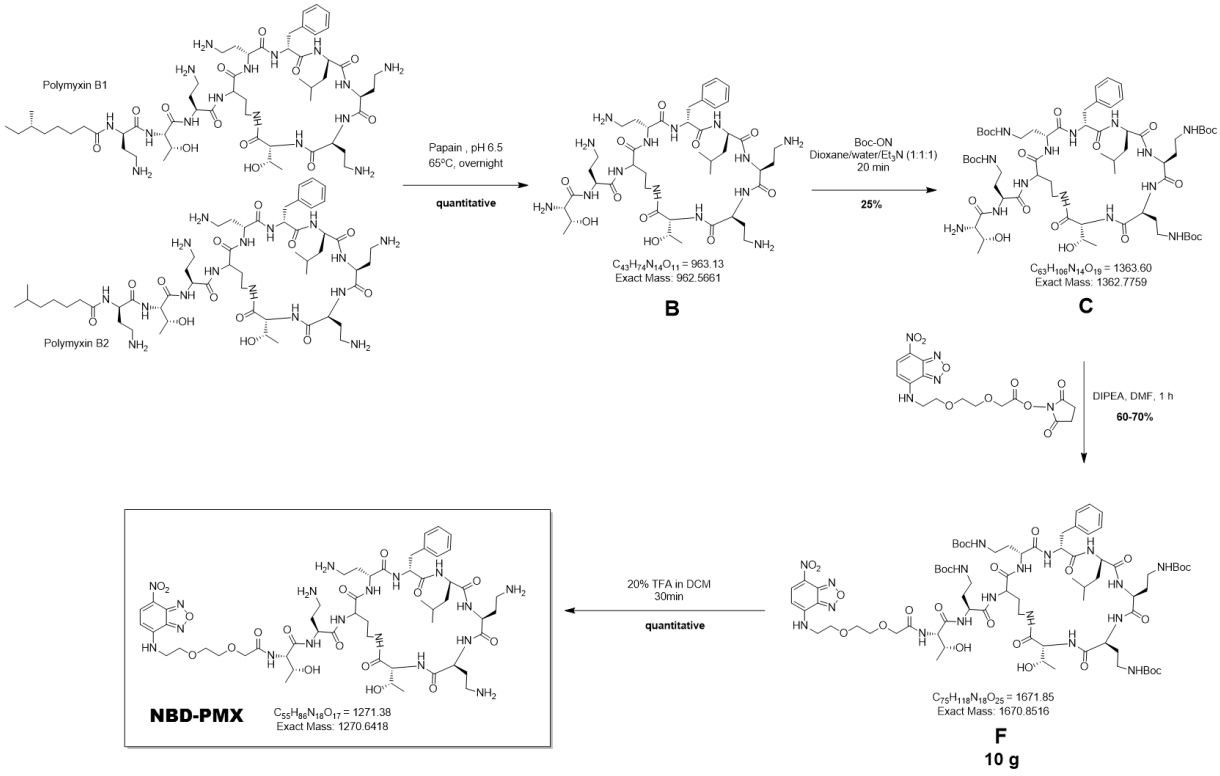
Supplementary Methods:

Haemolysis Assay: Erythrocytes were isolated from freshly drawn, anticoagulated human blood and resuspended to 20 vol % in PBS. In a 96-well microtiter plate, 100 µl of erythrocyte suspension was added to 100 µl of NBD-PMX at various concentrations. PBS acted as negative control and 0.2% Triton X-100 acted as positive control. The plate was incubated at 37°C for 1 h, each well was diluted with 150 µl of PBS and the plate centrifuged at 1,200g for 15 min. 100µl of the supernatant from each well was transferred to a fresh microtiter plate, and absorbance at 350 nm was measured in a Synergy H1 Multi-Mode Spectrophotometer (BioTek, VT, US). Data is expressed as % of positive control.

Murine intra-tracheal administration: Adult male CD-1 mice aged 8-12 weeks were given a single dose of NBD-PMX (100µg) in 50µl PBS or PBS vehicle control via direct intra-tracheal administration. Animals were monitored for clinical signs, weighed regularly and then sacrificed at 48 hours or 14 days (n=3 per group per time point). Following necroscopy, cellular infiltrate into the lung was assessed by harvesting bronchoalveolar lavage fluid (BALF) via 3 x 0.8ml PBS flushes of the lung and cytospin slides were prepared and stained with Diff-Quick (Thermo Fisher Scientific). Cell types were quantified using a light microscope. Lung, liver and kidney were taken for histopathological assessment, fixed and stained with haematoxylin and eosin.

Surfactant Constituent Synthesis: Surfactant 5µg 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 2.5µg L-α-Phosphatidyl-DL-glycerol sodium salt (from egg yolk lecithin; PG) were dissolved in 500µl chloroform and evaporated under nitrogen to a thin lipid film in a round bottom flask. The lipid film was rehydrated with PBS at 48°C for 1 hour with agitation (750rpm) to generate multilammelar vesicles (MLV) and diluted 1:4 for use in confocal experiments.

Chemical Synthesis of NBD-PMX: The NBD-PMX probe was synthesised from its precursor Polymyxin B sulfate in five steps (Scheme 1). The probe was synthesized using reported methods (49) with moderate modifications. The fluorophore was incorporated via an amide bond coupling using the NHS ester of the NBD-PEG2-OH and the tetra-boc protected polymyxin **C**. NBD-PMX was obtained after the TFA cleavage and HPLC purification.



Scheme 1. Synthetic route of NBD-PMX probe.

Experimental Procedure

Step 1: Preparation of B: Polymyxin B sulfate (10 g, 7.7 mmol, 1 eq) was dissolved in deionized water (200 mL) at a pH of 6.5 (use HCl aq solution to adjust the pH). Papain (1.5 g) was dissolved in water (25 mL) (same pH). The solutions were combined and toluene (0.5 mL) was added, and the mixture was gently stirred at 65 °C overnight. The mixture was then stirred in boiling water for 5 min and the precipitate formed (denatured papain) was removed by centrifugation and filtration. The filtrate was concentrated *in vacuo* and freeze dried to give the crude product **B** in quantitative yield. This step was carried forward to the next step without any further purification. MS *m*/*z* 963.2 (100%, $[M+H]^+$).

Step 2: Preparation of C: Crude **B** (5.5 g, 5.7 mmol, 1 eq) was dissolved in a mixture of H_2O: Dioxane: Et_3N (150 mL, 1:1:1) and Boc-ON (4.52 g, 17.1 mmol, 3eq) was added. The solution was stirred for 20 min at room temperature and then quenched with methanolic ammonia (20 mL, 2M ammonia in MeOH). The reaction was followed up by ELSD. Solvents were evaporated and the resulting mixture

was subjected to silica gel chromatography column (MeOH: DCM, 15:85) to afford white solid **B** (1.7 g, 22%). MS *m*/*z* 1363.7 (100%, $[M+H]^+$).

***Step 3: N*-(4-Nitrobenz-2-oxa-1,3-diazol-7-yl)amino-3,6-dioxaoctanoic acid (NBD-PEG$_2$-OH)**: DIEA (850 µl, 5.00 mmol) and solid 8-Amino-3,6-dioxaoctanoic acid (H_2N-PEG_2-OH) (392 mg, 2.40 mmol, 1 eq) were added slowly, over an hour, to a solution of NBD-Cl (401 mg, 2.01 mmol) in methanol (20 mL) at 0°C. The reaction mixture was stirred overnight at room temperature. The solvent was evaporated and the remaining material was purified by chromatography on silica with DCM/MeOH (8:2) as the eluent to give NBD-PEG2-OH (400 mg, 1.23 mmol, 51%) as dark red oil. ^{1}H NMR (500 MHz, DMSO): δ 10.9 (s, 1H; COOH), 8.49 (d, *J* = 8.5 Hz, 1H; CH NBD), 7.1 (s, 1H, NH), 6.23 (d, *J* = 8.5 Hz, 1H; CH NBD), 4.25 (s, 2H), 3.93 (t, *J* = 5.3 Hz, 2H; CH_2), 3.80 (s, 4H), 3.72 (t, *J* = 6.8 Hz, 2H; CH_2) ppm; MS (ESI-): m/z calcd for $C_{12}H_{14}N4O_7$ [M-H]: 325.1; found: 325.2

***N*-(4-Nitrobenz-2-oxa-1,3-diazol-7-yl)amino-3,6-dioxaoctanoic acid, succinimidyl ester (NBD-PEG$_2$-NHS):** To a solution of NBD-PEG_2-OH (2.4 g, 7.4 mmol, 1 eq) in anhydrous DCM (500 mL) was added EDC·HCl (1.56 g, 8.18 mmol, 1.1 eq) and DIPEA (1.36 mL, 10 mmol). After stirring the mixture for 10 min, *N*-hydroxysuccinimide (0.94 g, 8.18 mmol) was added and allowed to stir for 16 h. The reaction mixture was diluted with DCM (250 mL) and treated with 5% aqueous citric acid (2 x 200 mL), sat. aqueous $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4, filtered and reduced *in vacuo* to afford product as dark brown solid (1.0 g, quantitative). The crude was used for next step without further purification.

Step 4: Preparation of F: A solution of NBD-PEG_2-NHS (466 mg, 1.1 mmol, 1 eq), DIPEA (384 µL, 2.2 mmol, 2 eq) and amine **C** (1.5 g, 1.1 mmol, 1 eq) in DMF (150 mL) was stirred at room temperature for 1 h and protected from light. After completion of the reaction (TLC), volatiles are removed under vacuum. The crude mixture was purified by flash chromatography (DCM : MeOH, 90:10) to afford dark orange/brown solid (1.2 g, 65%). HPLC (254nm & 495nm) Rt = 7.80 min; *m*/*z* 1671.7 (25%, $[M+H]^+$); 1693.9 (65%, $[M+Na]^+$).

Step 5: *Preparation of* NBD-PMX: A solution of boc-protected polymyxin **F** (150 mg, 0.09 mmol) in 20% TFA in DCM (2 mL) was vigorously stirred for 45 min at room temperature and protected from light. The reaction mixture was evaporated in *vacuo* and the resultant was dissolved in ether. Ether layer was decanted after centrifugation (3 × 2 mL). The resultant yellow/brown solid (40 mg, quantitative) was dried under vacuum. The crude product was purified by preparative HPLC in MeOH/H_2O as gradient solvent system with 0.1% formic acid as an additive. The fractions collected from prep-HPLC were freeze dried to afford red/orange solid (30 mg, 26% recovery from HPLC).

Characterization: For analytical HPLC, a Poroshell 120 SB-C18, 2.7 µm, 4.6 x 50mm column was used with a diode array detector. For prep-HPLC method: Discovery C18 reverse-phase column (5 cm x 4.6 mm, 5 µm) with a flow rate of 1 mL/min and eluting with H_2O/MeOH/HCOOH (95/5/0.05) to H2O/MeOH/HCOOH (5/95/0.05), over 6 min, holding at 95% MeOH for 4 min, with detection at 254 and 495nm and by ELSD. HPLC (495nm): Rt = 4.1 min; MS *m/z* 1271.7 (95%, $[M+H]^+$); 1293.7 (100%, $[M+Na]^+$); FTMS calc. 636.3282 $([M+2H]/2)^+$, found 636.3344.

FTMS

Molecular formula: $C_{55}H_{96}N_{19}O_{17}$

Major peak detected: 636.3344 (*m/z*: 2)

Absorption / Emission: 467 nm / 539 nm.

Solubility: Fully soluble in water.

Stability: stable at room temperature for > than 1 week.

Storage: Stored at -20 °C under inert atmosphere. Protect from light.

Analytical HPLC of NBD-PMX

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                  STABILITY-010813.M)
Last changed    : 02/08/2013 06:31:29 PM by MARC
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                  in 6min, 1 min to 5% MeOH  and then 2min isocratic
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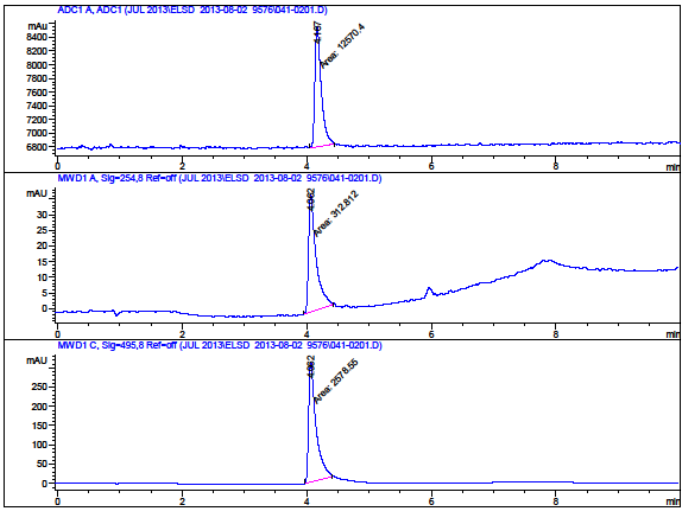

HRMS of NBD-PMX