Supporting Information

Early assessment and correlations of nanoclay's toxicity to their physical and chemical properties

Alixandra Wagner[a], Andrew P. White[a], Todd A. Stueckle[b], Derrick Banerjee[c], Konstantinos A. Sierros[c], Yon Rojanasakul[d], Sushant Agarwal[a], Rakesh K. Gupta[a], and Cerasela Zoica Dinu[a*]

[a]Department of Chemical and Biomedical Engineering, West Virginia University, Morgantown, WV 26506, USA
[b]National Institute for Occupational Safety and Health, Morgantown, WV 26505, USA
[c]Department of Mechanical and Aerospace Engineering, West Virginia University, Morgantown, WV 26506, USA
[d]Department of Basic Pharmaceutical Sciences, West Virginia University, Morgantown WV, 26506, USA

***Corresponding authors:**

Cerasela Zoica Dinu, Ph.D.
Department of Chemical and Biomedical Engineering
West Virginia University
Benjamin M. Statler College of Engineering and Mineral Resources
PO Box 6102
Morgantown, WV, 26506, USA
E-mail: cerasela-zoica.dinu@mail.wvu.edu
Tel.: +1 304 293 9338
Fax: +1 304 293 4139

Experimental

2.1 Dispersion analysis

The size distribution of the nanoclays and thermally degraded byproducts was determined by dynamic light scattering (DLS) via the Mastersizer 2000 with a Hydro 2000S accessory (Malvern Instruments). For this, samples of Nanomer PGV (PGV), an unmodified, hydrophilic bentonite, Nanomer I.31PS (I.31PS), a nanoclay surface modified with aminopropyltriethoxysilane at 0.5-5 wt. % and octadecylamine at 15-35 wt. %, Nanomer I.34TCN (I.34TCN), a nanoclay surface modified with methyl dihdroxyethyl hydrogenated tallow ammonium at 25-30 wt. % and, Nanomer I.44P (I.44P), a nanoclay surface modified with dimethyl dialkyl amine at 35-45 wt. %, as well as their thermally degraded byproducts (PGV900, I.31PS900, I.34TCN900, or I.44P900 respectively) were dispersed either in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) containing 5% fetal bovine serum (FBS), or in Small Airway Growth Medium (SAGM, Lonza) with SingleQuots Kit (Lonza) containing bovine pituitary extract, hydrocortisone, human Epidermal Growth Factor, epinephrine, transferrin, insulin, retinoic acid, triiodothyronine, gentamicin/amphotericin-B, and 1 % bovine serum albumin (BSA). Also, the nanoclays and byproducts were dispersed in a control, phosphate buffered saline (PBS, Lonza) and in distilled water containing 0.15 mg/ml Survanta®, a pulmonary surfactant. The solutions were then bath sonicated and dropped into the Hydro 2000S until laser obscuration was within 10-20%. The size analysis was performed 3 consecutive times with a stirrer speed of 1750 rpm and under continuous sonication in the Hydro 2000S accessory.

2.2 Cell Culture

Immortalized human bronchial epithelial cells (BEAS-2B) from American Type Culture Collection (ATCC) were cultured in 100 mm dishes (Corning, Inc.) in DMEM, containing 5% FBS, 1 % L-glutamine, and 1 % penicillin-streptomycin. The cells were incubated at 37 ^{0}C, 5 % CO_2, and in an 80 % relative humidity; consistent sub-culturing took place using 0.05 or 0.25 % trypsin (Invitrogen). Before each experiment, cells were grown to a monolayer of 90-100% confluency and cells in the 3rd-6th passage were used.

Additionally, small airway epithelial cells (SAECs) were cultured in SAGM with SingleQuots Kit and 1 % penicillin-streptomycin (Life Technologies). Cells were seeded into T-25 flasks (Corning, Inc.), grown to 75-80% confluency and subsequently split (5 passages total). All experiments completed with SAECs were performed using the same passage number.

2.3 Half Maximal Inhibitory Concentration (IC_{50})

BEAS-2B cells and SAECs were seeded into 12 well plates (Thermo Scientific) at densities of approximately $1.5x10^5$ and $2.0x10^5$ cells/ml, respectively. After 24 h, the cells were treated with PGV, I.31PS, I.34TCN, I.44P, or their thermally degraded byproducts at various doses ranging from 0 to 197 μg/cm^2. Before addition to the respective wells, each nanoclay or byproduct sample was sonicated for 10 min in a bath sonicator (2510 Branson; 100 W) with the concentrations used for exposure being serial dilutions from the original stock; cells in only media served as controls. After 24 h of exposure to individual treatment, the treated cells (as well as the controls) were washed to remove the nanoclays and byproducts, trypsinized, and stained with 0.4% trypan blue solution (Invitrogen). Subsequently, 10 μl of the sample containing the stained cells was added to

a hemocytometer, and the number of cells in the 4 outer grids was counted through the use of the Leica DM IL optical microscope (Leica Microsystems) and a 10X objective. Analyses of the cellular proliferation post-exposure were used to extrapolate IC_{50} values that would also be used in the remaining cellular assays.

2.4 Cellular Imaging

BEAS-2B cells and SAECs were seeded at densities of $1.5x10^5$ and $2.5x10^5$ cells/ml, respectively, in 24 well plates. After 24 h the cells were treated with the as-received nanoclays and thermally degraded byproducts, dispersed in media via a bath sonicator, at their respective, determined IC_{50} dose. After 24, 48, and 72 h of treatment the cells were imaged through use of a Leica DM IL optical microscope (Leica Microsystems) with a 10X objective. Two replicates were performed with 10 images, per replicate, taken at random spots within the well for each control and treatment.

2.5 Extracellular Reactive Oxygen Species (ROS)

BEAS-2B cells were seeded into 24 well plates at a density of approximately $1.5x10^5$ cells/ml. After 24 h, the cells were treated with nanoclays and byproducts dispersed in media through use of a bath sonicator at doses above and below their respective, determined IC_{50} value; cells exposed to only media served as control samples. After 24, 48, and 72 h of treatment, 50 µl of the media was transferred from the 24 well plate to its respective well in a black-bottomed 96 well plate (Corning, Inc.). Subsequently, 50 µl of PBS was added to each well in the 96 well plate. Fifty µl of the extracellular reactive oxygen species (ROS) assay reagent, Lumigen ECL Plus (Lumigen, Inc.), was also added to each well. The samples were subsequently incubated at room temperature for 5 min, in the dark before luminescence was evaluated using a FLUOstar OPTIMA plate reader (BMG LABTECH) at 600 nm. Media and treated media containing nanoclays or byproducts suspended in solution served as blanks. Respective cellular measurements of the samples were evaluated after subtracting the blanks in order to determine the effect treatment had on extracellular ROS. Four replicates were performed for each treatment.

Tables:

Table S1: Equations used to determine the percent moisture, high temperature volatiles, and ash present in the 4 Nanomer nanoclays upon thermal degradation via the TGA701 Thermogravimetric Analyzer.

Content	**Equation**
Moisture	(([Initial Mass]-[Moisture Mass])/[Initial Mass])*100
High Temperature Volatiles	(([Moisture Mass]-[Volatile Mass])/([Initial Mass])*100
Ash	([Ash Mass]/[Initial Mass])*100

Table S2: Elemental composition of as-received nanoclay and their thermally degraded byproducts as determined by EDX at 1 µm (n=10). The symbol * and ~ indicate significant differences between the unmodified nanoclay (PGV/PGV900) and the organically modified nanoclays and between the as-received nanoclay and its thermally degraded byproduct, respectively.

	PGV	I.31PS	I.34TCN	I.44P	PGV900	I.31PS900	I.34TCN900	I.44P900
Carbon	5.70 +/- 4.50	38.59 +/- 7.13*	26.87 +/- 6.70*	38.15 +/- 2.95*	2.33 +/- 5.55	22.67 +/- 16.31*~	13.68 +/- 13.41*~	14.88 +/- 13.92*~
Oxygen	39.67 +/- 2.62	28.49 +/- 3.59*	30.84 +/- 3.55*	23.89 +/- 3.76*	40.34 +/- 6.43	40.42 +/- 13.97~	44.28 +/- 4.63~	44.33 +/- 4.78~
Sodium	1.77 +/- 0.54	0.00 +/- 0.00*	0.00 +/- 0.00*	0.00 +/- 0.00*	2.11 +/- 0.41	0.00 +/- 0.00*	0.00 +/- 0.00*	0.00 +/- 0.00*
Magnesium	3.12 +/- 0.70	1.37 +/- 0.27*	1.46 +/- 0.33*	1.33 +/- 0.31*	4.01 +/- 0.69~	1.75 +/- 0.57*	1.74 +/- 0.47*	1.84 +/- 0.58*~
Aluminum	11.33 +/- 0.76	7.19 +/- 1.33*	10.98 +/- 1.53	9.70 +/- 1.28*	12.12 +/- 1.22	7.86 +/- 2.48*	11.39 +/- 3.33	12.57 +/- 5.01
Silicon	32.93 +/- 4.20	21.21 +/- 4.34*	26.52 +/- 4.34*	24.07 +/- 4.01*	33.14 +/- 4.29	22.22 +/- 7.87*	26.90 +/- 9.82	24.50 +/- 6.33*
Calcium	1.70 +/- 0.77	0.00 +/- 0.00*	0.00 +/- 0.00*	0.00 +/- 0.00*	1.85 +/- 0.98	0.00 +/- 0.00*	0.00 +/- 0.00*	0.29 +/- 0.76*
Iron	3.79 +/- 1.18	2.27 +/- 0.69*	3.62 +/- 1.00	2.86 +/- 0.73*	4.02 +/- 2.83	1.29 +/- 0.51*~	2.02 +/- 1.30~	1.59 +/- 0.50*~
Chlorine	0.00 +/- 0.00	0.78 +/- 0.46*	0.00 +/- 0.00	0.00 +/- 0.00	0.00 +/- 0.00	0.00 +/- 0.00~	0.00 +/- 0.00	0.00 +/- 0.00
Potassium	0.00 +/- 0.00	0.12 +/- 0.38	0.00 +/- 0.00	0.00 +/- 0.00	0.00 +/- 0.00	0.20 +/- 0.24*	0.00 +/- 0.00	0.00 +/- 0.00

Table S3: Average particle size (μm) of <90 % of the four as-received nanoclays and their thermally degraded byproducts in solutions of PBS, DMEM, SAGM, or Survanta with +/- standard deviation (n=3). The symbol * and ~ indicate significant differences between the unmodified nanoclay (PGV/PGV900) and the organically modified nanoclays and between the as-received nanoclay and its thermally degraded byproduct, respectively

	PGV	**I.31PS**	**I.34TCN**	**I.44P**	**PGV900**	**I.31PS900**	**I.34TCN900**	**I.44P900**
PBS	9.17 +/- 1.59	0.71 +/- 0.05*	0.75 +/- 0.06*	7.83 +/- 0.44	3.72 +/- 5.70	8.28 +/- 0.19~	9.71 +/- 0.01~	10.99 +/- 0.15~
DMEM	8.55 +/- 1.0	0.85 +/- 0.04*	0.92 +/- 0.04*	7.69 +/- 0.02	11.54 +/- 0.09~	8.23 +/- 0.02*~	9.42 +/- 0.36*~	8.85 +/- 0.01*~
SAGM	0.15 +/- 0.02	0.85 +/- 0.01*	0.85 +/- 0.05*	7.73 +/- 0.05*	9.02 +/- 0.34~	6.71 +/- 0.01*~	8.55 +/- 0.01~	10.04 +/- 0.12*~
Survanta	0.12 +/- 0.01	7.79 +/- 0.12*	7.46 +/- 0.35*	8.12 +/- 0.17*	8.03 +/- 0.33~	8.87 +/- 0.21*~	10.33 +/- 0.65*~	10.86 +/- 0.33*~

Table S4: Average particle size (μm) distributions of the 4 as-received nanoclays and their thermally degraded byproducts in PBS with +/- standard deviation (n=3). The symbol * and ~ indicate significant differences between the unmodified nanoclay (PGV/PGV900) and the organically modified nanoclays and between the as-received nanoclay and its thermally degraded byproduct, respectively.

	PGV	**I.31PS**	**I.34TCN**	**I.44P**	**PGV900**	**I.31PS900**	**I.34TCN900**	**I.44P900**
<10%	3.04 +/-0.38	0.33 +/- 0.02*	0.38 +/- 0.03*	2.81 +/- 0.12	1.22 +/- 1.79	2.87 +/- 0.02~	3.15 +/- 0.00~	3.62 +/- 0.02~
<50%	4.78 +/- 0.72	0.44 +/- 0.03*	0.49 +/- 0.03*	4.11 +/- 0.22	1.88 +/- 2.82	4.30 +/- 0.06~	4.91 +/- 0.00~	5.81 +/- 0.06~
<90%	9.17 +/- 1.59	0.71 +/- 0.05*	0.75 +/- 0.06*	7.83 +/- 0.44	3.72 +/- 5.70	8.28 +/- 0.19~	9.71 +/- 0.01~	10.99 +/- 0.15~

Table S5: Average particle size (μm) distributions of the 4 as-received nanoclays and their thermally degraded byproducts in DMEM with +/- standard deviation (n=3). The symbol * and ~ indicate significant differences between the unmodified nanoclay (PGV/PGV900) and the organically modified nanoclays and between the as-received nanoclay and its thermally degraded byproduct, respectively.

	PGV	**I.31PS**	**I.34TCN**	**I.44P**	**PGV900**	**I.31PS900**	**I.34TCN900**	**I.44P900**
<10%	2.55 +/- 0.14	0.38 +/- 0.02*	0.44 +/- 0.02*	2.84 +/- 0.00*	4.06 +/- 0.01~	2.95 +/- 0.00*~	3.21 +/- 0.10*~	3.25 +/- 0.00*~
<50%	3.81 +/1 0.27	0.50 +/- 0.02*	0.57 +/- 0.02*	4.09 +/- 0.01	6.11 +/1 0.05~	4.28 +/- 0.00*~	4.89 +/- 0.20*~	4.83 +/- 0.01*~
<90%	8.55 +/- 1.0	0.85 +/- 0.04*	0.92 +/- 0.04*	7.69 +/- 0.02	11.54 +/- 0.09~	8.23 +/- 0.02*~	9.42 +/- 0.36*~	8.85 +/- 0.01*~

Table S6: Average particle size (μm) distributions of the 4 as-received nanoclays and their thermally degraded byproducts in SAGM with +/- standard deviation (n=3). The symbol * and ~ indicate significant differences between the unmodified nanoclay (PGV/PGV900) and the organically modified nanoclays and between the as-received nanoclay and its thermally degraded byproduct, respectively.

	PGV	**I.31PS**	**I.34TCN**	**I.44P**	**PGV900**	**I.31PS900**	**I.34TCN900**	**I.44P900**
<10%	0.05 +/- 0.01	0.37 +/- 0.00*	0.41 +/- 0.01*	2.75 +/- 0.02*	3.07 +/- 0.08~	2.52 +/- 0.00*~	2.83 +/- 0.00*~	3.28 +/- 0.01*~
<50%	0.08 +/- 0.02	0.50 +/- 0.02*	0.53 +/- 0.02*	4.03 +/- 0.02*	4.66 +/- 0.19~	3.61 +/- 0.01*~	4.23 +/- 0.00*~	5.11 +/- 0.03*~
<90%	0.15 +/- 0.02	0.85 +/- 0.01*	0.85 +/- 0.05*	7.73 +/- 0.05*	9.02 +/- 0.34~	6.71 +/- 0.01*~	8.55 +/- 0.01~	10.04 +/- 0.12*~

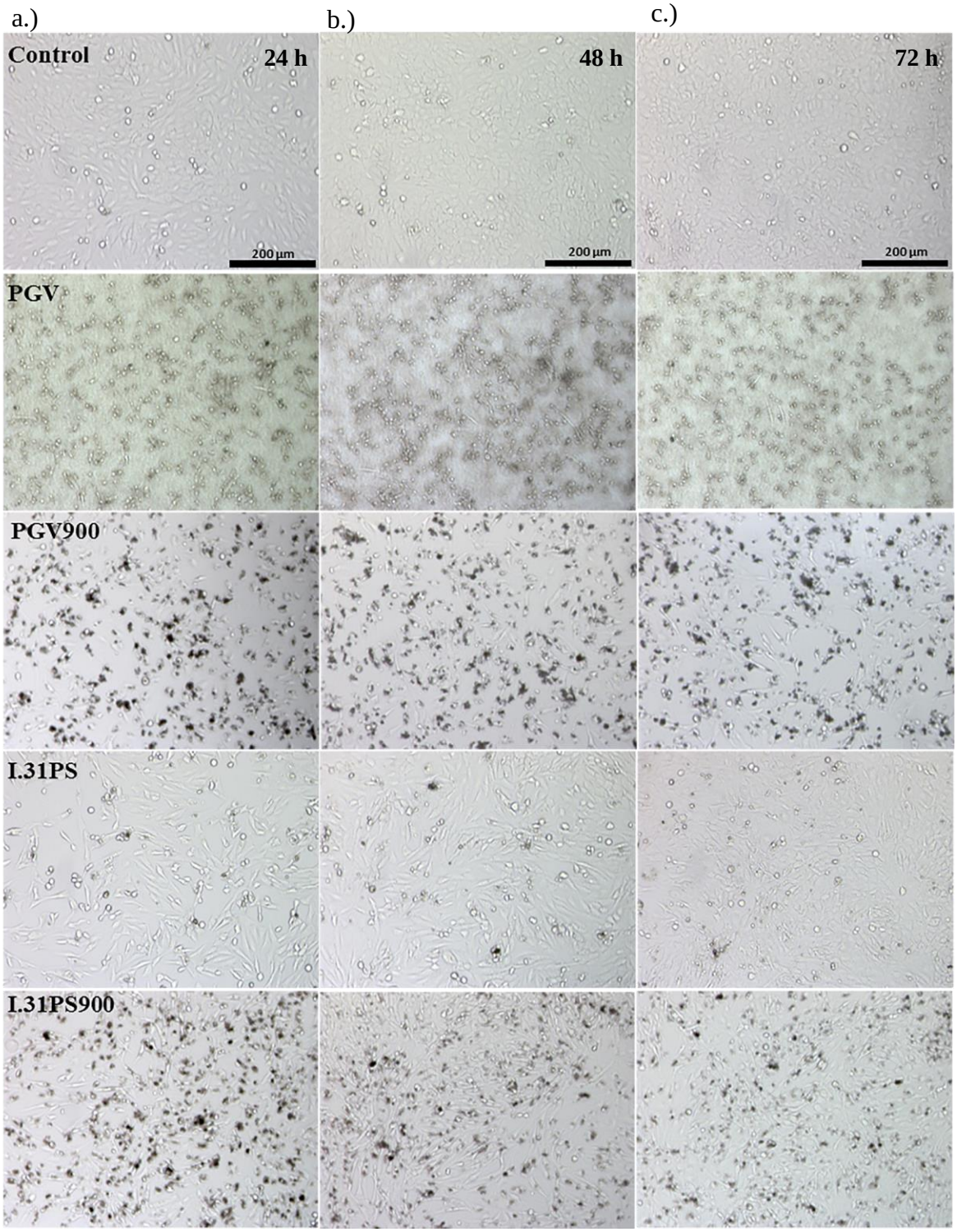
Table S7: Average particle size (μm) distributions of the 4 as-received nanoclays and their thermally degraded byproducts in Survanta with +/- standard deviation (n=3). The symbol * and ~ indicate significant differences between the unmodified nanoclay (PGV/PGV900) and the organically modified nanoclays and between the as-received nanoclay and its thermally degraded byproduct, respectively.

	PGV	**I.31PS**	**I.34TCN**	**I.44P**	**PGV900**	**I.31PS900**	**I.34TCN900**	**I.44P900**
<10%	0.03 +/- 0.00	2.42 +/- 0.16*	2.36 +/- 0.11*	2.64 +/- 0.02*	3.39 +/- 0.19~	2.94 +/- 0.02*~	3.35 +/- 0.21~	4.10 +/- 0.10*~
<50%	0.06 +/- 0.00	3.78 +/- 0.25*	3.63 +/- 0.19*	3.99 +/- 0.05*	4.94 +/1 0.25~	4.44 +/- 0.04*~	5.49 +/- 0.41~	6.34 +/- 0.10*~
<90%	0.12 +/- 0.01	7.79 +/- 0.12*	7.46 +/- 0.35*	8.12 +/- 0.17*	8.03 +/- 0.33~	8.87 +/- 0.21*~	10.33 +/- 0.65*~	10.86 +/- 0.33*~

Table S8: IC_{50} values ($\mu g/cm^2$) of BEAS-2B cells and SAECs treated with as-received nanoclays and thermally degraded byproducts.

	BEAS-2B	SAECs
PGV	60.3	13.2
I.31PS	4.5	1.3
I.34TCN	2.1	0.5
I.44P	13.7	14.2
PGV900	96.3	26.3
I.31PS900	43.2	21.8
I.34MN900	51.1	21.6
I.44P900	42.9	8.7

Figures:


a.)
b.)
c.)
Control
24 h
48 h
72 h
200 µm
200 µm
200 µm
PGV
PGV900
I.31PS
I.31PS900

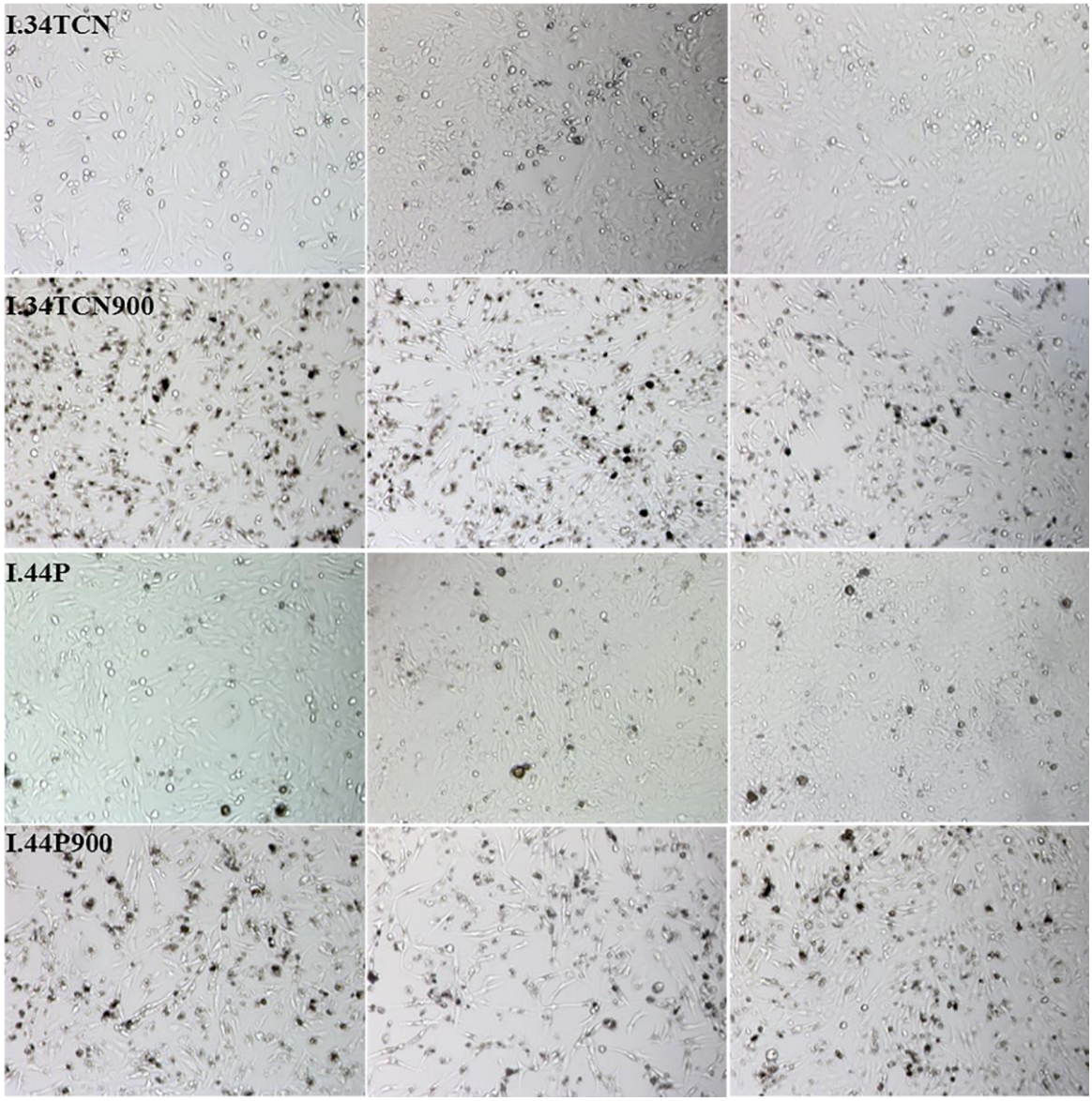
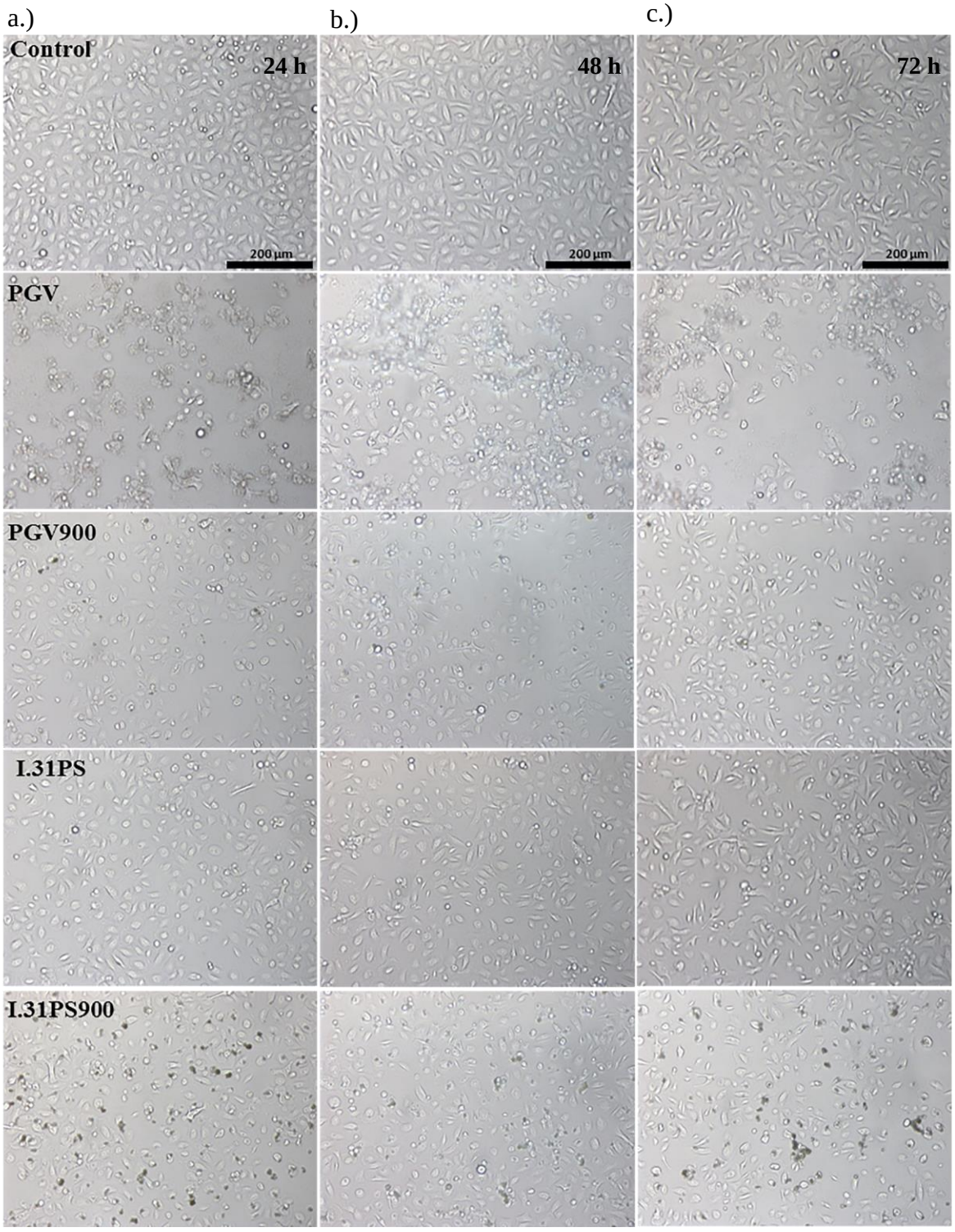


Figure S1: Representative optical images of BEAS-2B cells treated with as-received Nanomer nanoclays and thermally degraded byproducts at their respective IC_{50} doses at a.) 24 h, b.) 48 h, and c.) 72 h post-treatment (n=2).


a.)
b.)
c.)
Control
24 h
48 h
72 h
200 µm
200 µm
200 µm
PGV
PGV900
I.31PS
I.31PS900

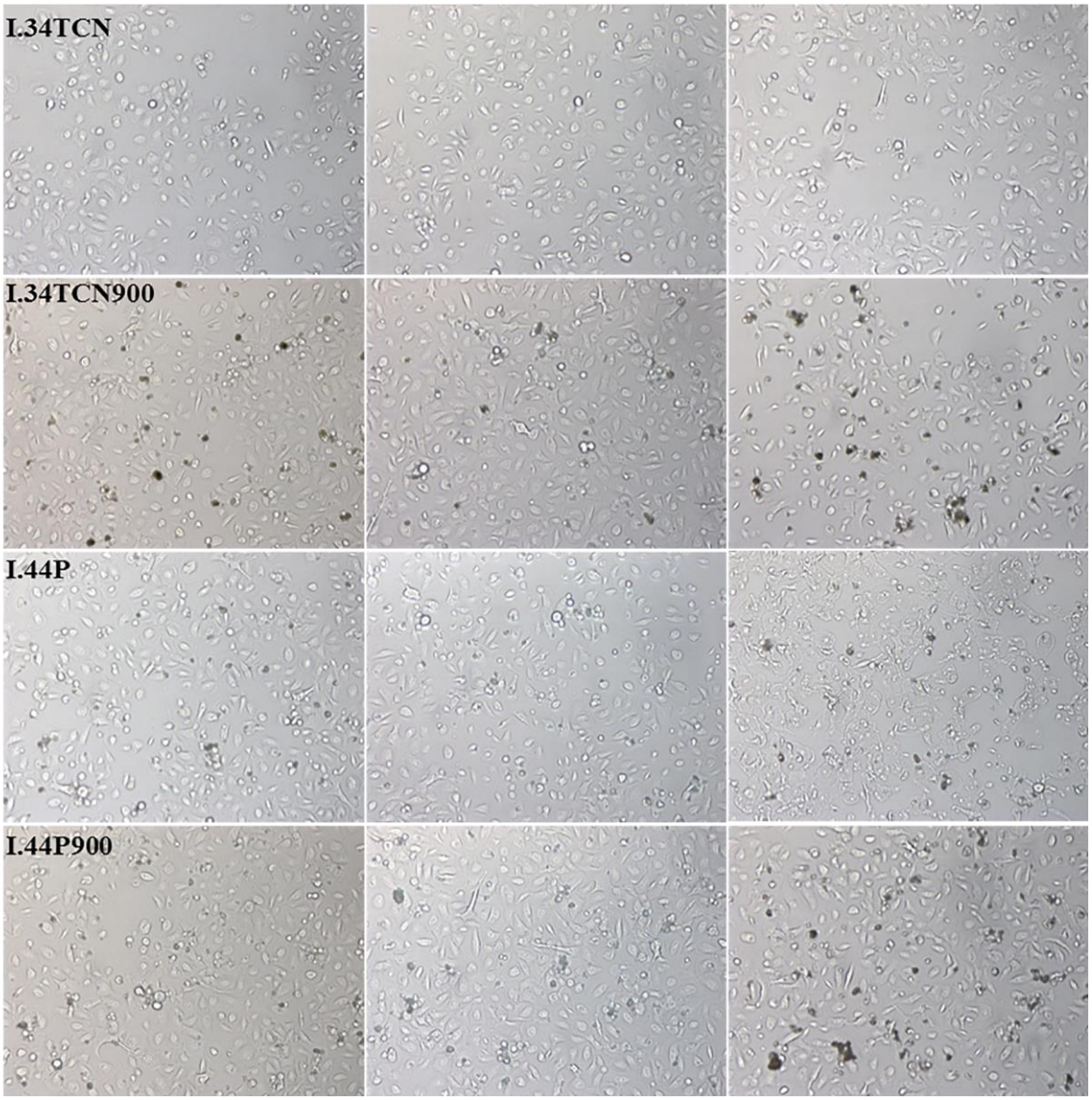
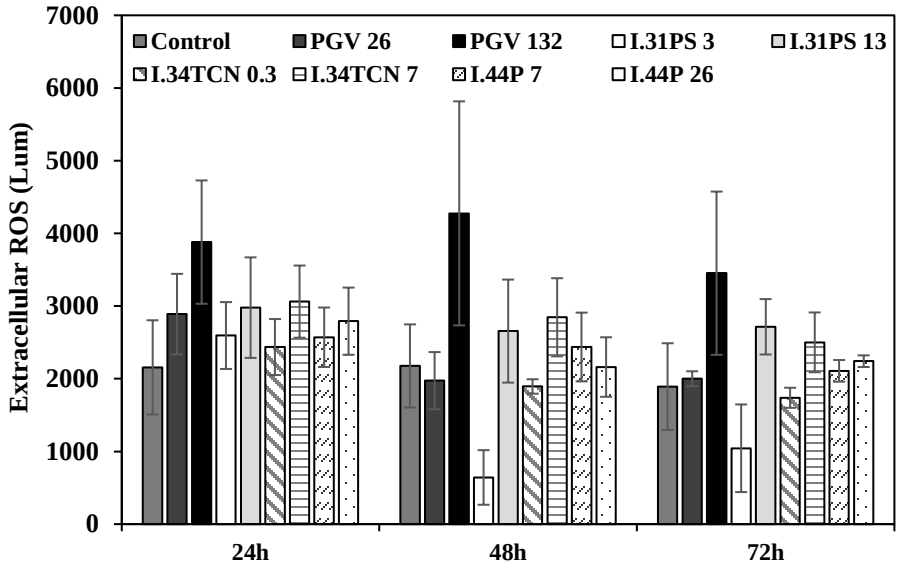


Figure S2: Representative optical images of SAECs treated with as-received Nanomer nanoclays and thermally degraded byproducts at their respective IC_{50} doses at a.) 24 h, b.) 48 h, and c.) 72 h post-treatment (n=2).

a.)



b.)

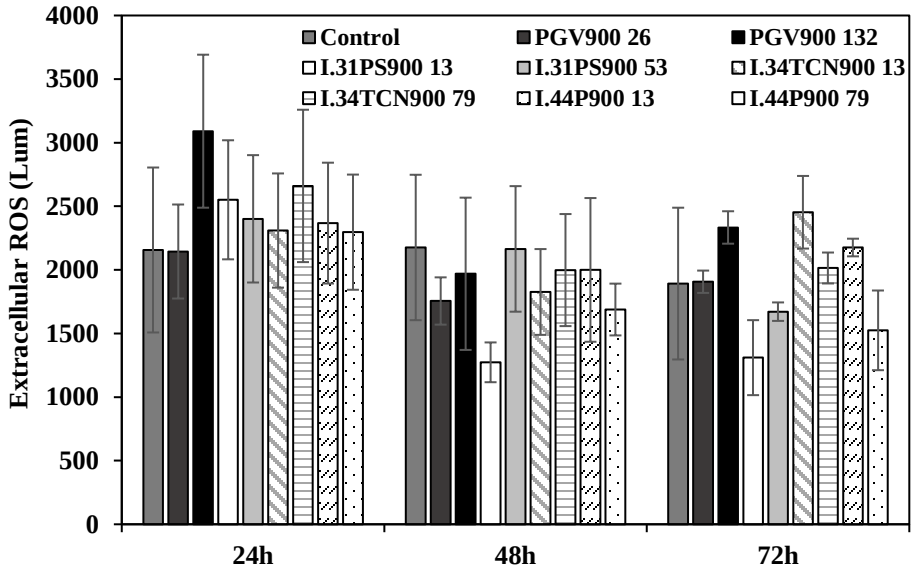


Figure S3: Extracellular ROS production by BEAS-2B cells after treatment with a.) as-received nanoclays above and below their respective IC_{50} dose and b.) thermally degraded byproducts above and below their respective IC_{50} dose over 72 h (n=4).

List of Abbreviations

MMT: montmorillonite

PGV: as-received Nanomer PGV

I.31PS: as-received Nanomer I.31PS

I.34TCN: as-received Nanomer I.34TCN

I.44P: as-received Nanomer I.44P

PGV900: thermally degraded Nanomer PGV

I.31PS900: thermally degraded Nanomer I.31PS

I.34TCN900: thermally degraded Nanomer I.34TCN

I.44P900: thermally degraded Nanomer I.44P

BEAS-2B cells: immortalized human bronchial epithelial cells

SAECs: small airway epithelial cells

DMEM: Dulbecco's Modified Eagle Medium (cellular media for BEAS-2B cells)

SAGM: Small Airway Growth Medium (cellular media for SAECs)

PBS: phosphate buffered saline

ROS: reactive oxygen species