Supporting information

Dry-powder formulations of non-covalent protein complexes with linear or miktoarm copolymers for pulmonary delivery

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Supplementary data

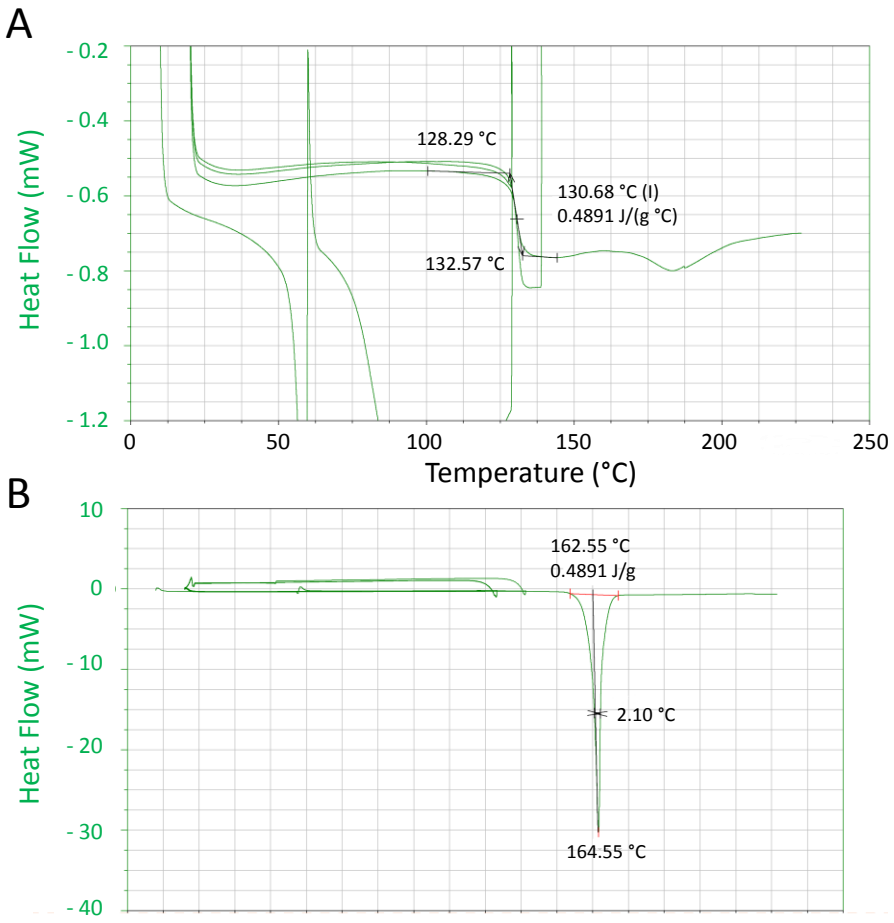

Figure S1. Representative DSC thermograms of dry powder formulations. A). 5 % free lysozyme (w/w), 6.6 % (*w/w*) phosphate buffer salts, 10 % leucine, 78.4 % (*w/w*) trehalose; and B). 5 % free lysozyme (*w/w*), 6.6 % (*w/w*) phosphate buffer salts, 10 % leucine, 78.4 % (*w/w*) mannitol.

Table S1. Composition of dry powder formulations used in this study.

Formulation	Trehalose (%*w/w*)	Mannitol (%*w/w*)	Leucine (%*w/w*)	Phosphate buffer salts (%*w/w*)	Nanocomplexes (*w/w*) (Lysozyme (*w/w*))
Up to 100% Trehalose	93.4	-	-	6.6	no lysozyme
	88.4	-	-	6.6	5% Free lysozyme
	83.4	-	-	6.6	10% ($mPEG_{2k}$-*lin*-GA_{10})-Lysozyme (4.9% Lysozyme)
	83.4	-	-	6.6	10% ($mPEG_{2k}$-*lin*-GA_{30})-Lysozyme (5.9% Lysozyme)
	83.4	-	-	6.6	10% ($mPEG_{2k}$-*mik*-$(GA_{10})_3$)-Lysozyme (5.9% Lysozyme)
	83.4	-	-	6.6	10% ($mPEG_{2k}$-*mik*-$(GA_{30})_3$)-Lysozyme (6.6% Lysozyme)
Up to 100% Trehalose + 10 % Leucine	83.4	-	10	6.6	no lysozyme
	78.4	-	10	6.6	5% Free lysozyme
	73.4	-	10	6.6	10% ($mPEG_{2k}$-*lin*-GA_{10})-Lysozyme (4.9% Lysozyme)
	73.4	-	10	6.6	10% ($mPEG_{2k}$-*lin*-GA_{30})-Lysozyme (5.9% Lysozyme)
	73.4	-	10	6.6	10% ($mPEG_{2k}$-*mik*-$(GA_{10})_3$)-Lysozyme (5.9% Lysozyme)
	73.4	-	10	6.6	10% ($mPEG_{2k}$-*mik*-$(GA_{30})_3$)-Lysozyme (6.6% Lysozyme)
Up to 100% Mannitol	-	93.4	-	6.6	no lysozyme
	-	88.4	-	6.6	5% Free lysozyme
	-	83.4	-	6.6	10% ($mPEG_{2k}$-*lin*-GA_{10})-Lysozyme (4.9% Lysozyme)
	-	83.4	-	6.6	10% ($mPEG_{2k}$-*lin*-GA_{30})-Lysozyme (5.9% Lysozyme)
	-	83.4	-	6.6	10% ($mPEG_{2k}$-*mik*-$(GA_{10})_3$)-Lysozyme (5.9% Lysozyme)
	-	83.4	-	6.6	10% ($mPEG_{2k}$-*mik*-$(GA_{30})_3$)-Lysozyme (6.6% Lysozyme)
Up to 100% Mannitol + 10% Leucine	-	83.4	10	6.6	no lysozyme
	-	78.4	10	6.6	5% Free lysozyme
	-	73.4	10	6.6	10% ($mPEG_{2k}$-*lin*-GA_{10})-Lysozyme (4.9% Lysozyme)
	-	73.4	10	6.6	10% ($mPEG_{2k}$-*lin*-GA_{30})-Lysozyme (5.9% Lysozyme)
	-	73.4	10	6.6	10% ($mPEG_{2k}$-*mik*-$(GA_{10})_3$)-Lysozyme (5.9% Lysozyme)
	-	73.4	10	6.6	10% ($mPEG_{2k}$-*mik*-$(GA_{30})_3$)-Lysozyme (6.6% Lysozyme)

Table S2. Moisture content of dry powders investigated in this study. All formulations contained 6.6 % (*w/w*) phosphate buffer salts.

Excipients (*w/w*)	**Nanocomplexes (% *w/w*)**	**Moisture content (%)**
Up to 100% Trehalose	5% Free lysozyme	2.0
	10% (mPEG$_{2k}$-*lin*-GA$_{10}$)- lysozyme	5.0
	10% (mPEG$_{2k}$-*lin*-GA$_{30}$)- lysozyme	2.1
	10% (mPEG$_{2k}$-*mik*-(GA$_{10}$)$_3$)- lysozyme	2.3
	10% (mPEG$_{2k}$-*mik*-(GA$_{30}$)$_3$)- lysozyme	2.4
Up to 100% Trehalose + 10% Leucine	5% Free lysozyme	2.3
	10% (mPEG$_{2k}$-*lin*-GA$_{10}$)- lysozyme	1.8
	10% (mPEG$_{2k}$-*lin*-GA$_{30}$)- lysozyme	2.4
	10% (mPEG$_{2k}$-*mik*-(GA$_{10}$)$_3$)- lysozyme	2.9
	10% (mPEG$_{2k}$-*mik*-(GA$_{30}$)$_3$)- lysozyme	2.8
Up to 100% Mannitol	5% Free lysozyme	0.9
	10% (mPEG$_{2k}$-*lin*-GA$_{10}$)- lysozyme	0.9
	10% (mPEG$_{2k}$-*lin*-GA$_{30}$)- lysozyme	1.7
	10% (mPEG$_{2k}$-*mik*-(GA$_{10}$)$_3$)- lysozyme	1.0
	10% (mPEG$_{2k}$-*mik*-(GA$_{30}$)$_3$)- lysozyme	0.9
Up to 100% Mannitol + 10% Leucine	5% Free lysozyme	0.7
	10% (mPEG$_{2k}$-*lin*-GA$_{10}$)- lysozyme	0.5
	10% (mPEG$_{2k}$-*lin*-GA$_{30}$)- lysozyme	1.4
	10% (mPEG$_{2k}$-*mik*-(GA$_{10}$)$_3$)- lysozyme	0.6
	10% (mPEG$_{2k}$-*mik*-(GA$_{30}$)$_3$)- lysozyme	1.7

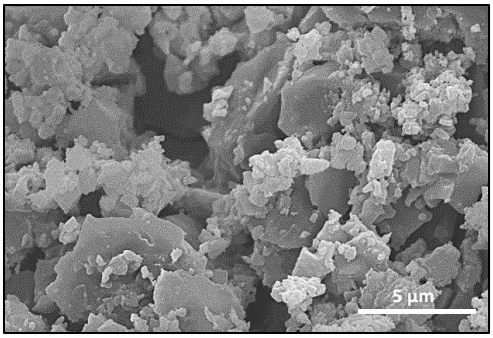

Figure S2. Representative scanning electron microscopy (SEM) images of dry powder formulation containing lysozyme-(mPEG$_{2k}$-*mik*-(GA$_{30}$)$_3$) nanocomplexes. Excipients: trehalose and phosphate buffer salts. Control uncomplexed lysozyme-based dry powders containing trehalose and phosphate buffer salts were not analysed by SEM due to low amount of material recovered after spray-drying.

Metabolic activity (MTS assay)

A549 or Calu-3 cells were seeded in 96-well plates at 2-3.10^4 cells *per* well and permitted to grow for 24 h. Medium was then removed, cells washed with phosphate buffer and samples, at polymer concentrations ranging from 25 to 200 µg mL^{-1}, applied to the cells (200 µl per well) for 4 or 24 hours in growth buffer. After that, medium was removed, cells washed with phosphate buffer and 120 µL of MTS solution (20 µL MTS + 100 µL growth medium) were added. After 3 hours incubation, the optical density was read at 490 nm. Experiments were carried out in triplicate. Experiments were carried out in triplicate.

Controls for both experiments were performed using 4% (*v/v*) Triton X-100 and untreated cells, indicating 0% metabolic activity (positive control) and 100% metabolic activity (negative control), respectively. The metabolic activity was calculated using the following formula:

$$\%\ Metabolic\ activity = \frac{\mathrm{S} - \mathrm{P}}{\mathrm{N} - \mathrm{P}}\ \mathrm{X}\ 100$$

where S, N and P are the absorbance of treated cells, negative control and positive control, respectively.

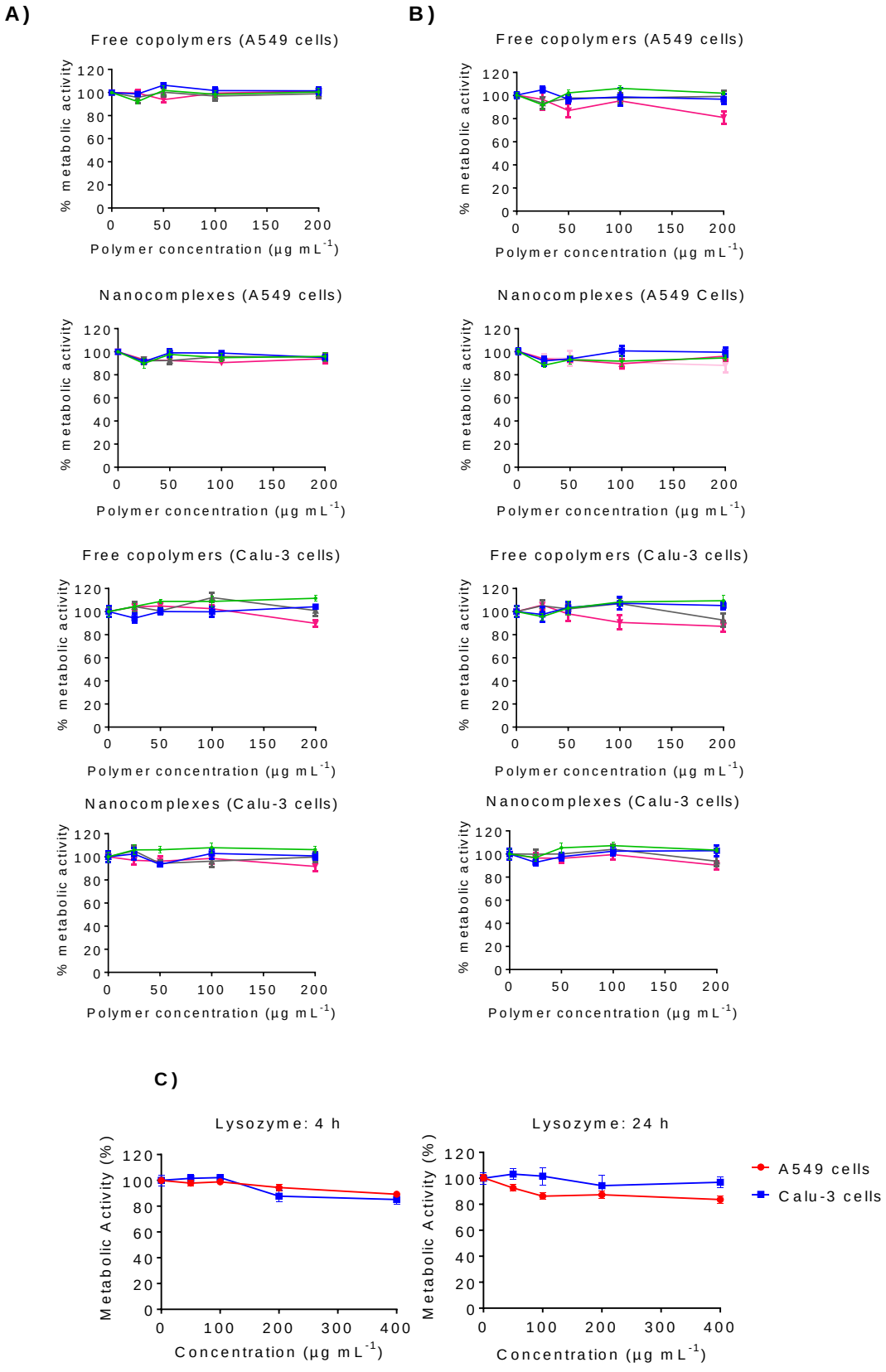
A)
B)
Free copolymers (A549 cells)
Nanocomplexes (A549 cells)
Free copolymers (Calu-3 cells)
Nanocomplexes (Calu-3 cells)
% metabolic activity
Polymer concentration (µg mL-1)
C)
Lysozyme: 4 h
Lysozyme: 24 h
Metabolic Activity (%)
Concentration (µg mL-1)
A549 cells
Calu-3 cells

Figure S3. MTS assay in A549 and Calu-3 cells after treatment with free $mPEG_{2k}$-polyGA copolymers and ($mPEG_{2k}$-polyGA)-lysozyme nanocomplexes at various concentrations. A) 4 h and B) 24 h exosure. ($mPEG_{2k}$-*lin*-GA_{10}) (**green**), ($mPEG_{2k}$-*lin*-GA_{30}) (**blue**), ($mPEG_{2k}$-*mik*-$(GA_{10})_3$) (**grey**), $mPEG_{2k}$-*mik*-$(GA_{30})_3$ (**pink**)-derived complexes or copolymer. C) MTS assay in A549 and Calu-3 cells after 4 and 24 hours treatment with un-complexed, 'free' lysozyme in solution.

LDH assay on cell membrane integrity

A549 or Calu-3 cells were seeded in 96-well plates at 2-3X10^4 cells *per* well and permitted to grow for 24 hours. Medium was then removed, cells washed with phosphate buffer and samples, at polymer concentrations ranging from 25 to 200 µg mL^{-1}, added to the cells (200 µl per well) for 4 hours. After that, the plate was centrifuged 4 min at 1,500 rpm, 50 µL of supernatant were disposed in a new 96-well plate and 100 µL of a LDH kit solution were added according to the manufacturer's instructions (*in vitro* toxicology assay kit, lactic dehydrogenase based, Sigma Aldrich). The mixture was incubated during 30 min at room temperature. After that, 15 µL of 1 M HCl were added to quench the reaction. The absorbance was measured at 490 nm. Controls were performed using 4% (*v/v*) Triton X-100 and untreated cells indicating 100% LDH release (positive control) and 0% LDH release (negative control) respectively. Experiments were carried out in triplicate. The percentage of LDH release was calculated using the following formula:

$$\%\ \text{LDH Release} = \frac{\text{S} - \text{N}}{\text{P} - \text{N}}\ \text{x}\ 100$$

where S, N and P are the absorbance of treated cells, negative control and positive control, respectively.

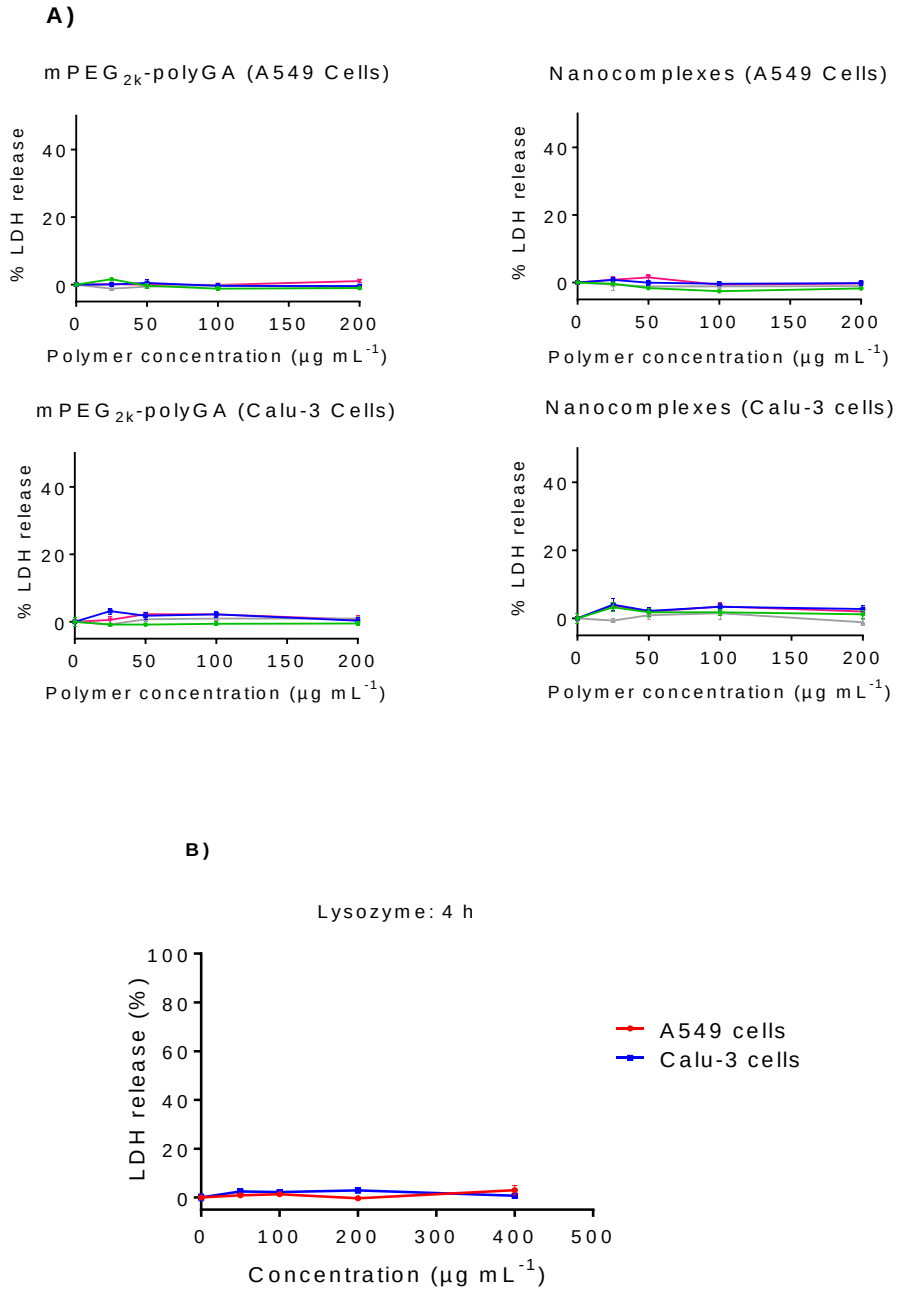

Figure S4. Percentage of total LDH released from A549 and Calu-3 cells. **A)** Cell treated with free mPEG$_{2k}$-polyGA copolymers and (mPEG$_{2k}$-polyGA)-lysozyme nanocomplexes for 4 h. mPEG$_{2k}$-*lin*-GA$_{10}$ (**green**), mPEG$_{2k}$-*lin*-GA$_{30}$ (**blue**), mPEG$_{2k}$-*mik*-(GA$_{10}$)$_3$ (**grey**), mPEG$_{2k}$-*mik*-(GA$_{30}$)$_3$ (**pink**)-derived complexes or copolymer. **B)** Percentage of total LDH released from A549 and Calu-3 cells after 4 hours treatment with un-complexed, 'free' lysozyme in solution.