Preserving catalytic activity and enhancing biochemical stability of the therapeutic enzyme asparaginase by biocompatible multilayered polyelectrolyte microcapsules.

Christos S. Karamitros[1], Alexey M. Yashchenok[2], Helmuth Möhwald[2], Andre G. Skirtach[2,3], Manfred Konrad[1]*

Supplementary Information

The open reading frame (ORF) coding for yeast cytoplasmic L-asparaginase *Sc*ASNaseI (UniProt P38986, gene ASP1) consisting of 1143 bp, was amplified with PCR using as template genomic DNA from the *Saccharomyces cerevisiae* S288C wildtype strain. NdeI and BamHI sites were incorporated at the 5'- and 3'-oligonucleotides' ends, respectively: 5'-oligo GGAATTCCATATGAAAAGCGATTCAGTTGAAATC; 3'-oligo CGCGGATCCTCACCCACCATAGACGCCAGTG. The PCR reaction mixture consisted of oligonucleotide mix, KAPA high fidelity buffer, dNTPs and KAPA HiFi DNA polymerase. The reaction was initiated at 95 □C for 3 min, followed by 25 cycles of denaturation at 98 □C for 20 s, primer annealing at 60 □C for 30 s, and extension at 72 □C for 30 s. The amplification reaction was terminated after a 5 min polishing step at 72 □C. The PCR product (**Figure S1**) was gel-purified, digested with NdeI and BamHI H.F., purified with PCR clean-up kit and then ligated overnight at 16 □C into pET14b-SUMO vector (**Figure S2**) using T4 DNA ligase. The

ligation mixture was used to transform DH5α *E.coli* cells. Positive clones were determined following colony PCR screening using one primer for the vector (Forward) and one primer for the insert (Reverse), restriction digestion with NdeI and BamHI H.F., and finally sequencing of the cloned DNA insert. The expressed protein construct includes an N-terminal 6-histidine tag, followed by the yeast SUMO (Small Ubiquitin MOdifier) tag which has proven to improve heterologous protein solubility and stability.

E.coli BL21(DE3) C41 cells containing the *Sc*ASNaseI-encoding plasmid were cultured overnight at 37 □C in TB medium supplemented with 200 µg/ml ampicillin. A fraction of this culture was used to inoculate fresh TB culture (dilution 1:100) supplemented with 200 µg/ml ampicillin. When $O.D._{600}$ reached ~ 0.5-0.7, the expression was induced by adding IPTG to a final concentration of 1 mM. After incubation at 37 □C for 8 h, the culture was centrifuged at 4,000g for 30 min, the cells were harvested, resuspended in affinity matrix binding buffer (50 mM Na_2HPO_4, 0.5 M NaCl, 10 mM imidazole, pH 8.0), and ultimately lysed by sonication. The cell lysate was centrifuged at 17,200g for 45 min, the resulting supernatant mixed with pre-equilibrated nickel agarose beads, and incubated at 4 □C for 3 h under rotation. Subsequently, the mixture was filled in a 5 mL polypropylene column and dried by gravity. The nickel resin was washed with 25 bed volumes of washing buffer (50 mM Na_2HPO_4, 1 M NaCl, 20 mM imidazole, pH 8.0). Finally, the bound protein was eluted from the column by applying 300 mM imidazole, and dropwise collection of fractions. All purification steps were performed at 4 □C. The collected fractions were mixed, and buffer was exchanged against 50 mM Na_2HPO_4, 0.5 NaCl, pH 7.5 using a PD-10 column (GE). The eluted protein fraction was incubated with yeast SUMO protease (molar ratio protease:protein ~ 1:100) at 30 □C for 2 h in order to cleave the N-terminal His_6-SUMO tag. In the last purification step, the protein was subjected to size exclusion

chromatography by passing it through a Superdex 200 column (Pharmacia/GE) to remove the cleaved tag. Protein purity was evaluated by SDS-PAGE and was estimated to exceed 95%. The protein sample was aliquoted, mixed with 25% glycerol, and stored at -20 □C until use. The *E.coli* L-ASNase II (*Ec*ASNaseII) was expressed and purified analogously.

The fusion protein eGFP-*Sc*ASNaseI was constructed using a modified pET14b vector which was generated for the needs of the present study (**Figure S3**). The N-terminus included the His_6-eGFP part followed by a short hydrophilic flexible linker consisting of $(Gly\text{-}Ser)_2$. Downstream of the His_6-eGFP plus linker moiety, the coding region for *Sc*ASNaseI was inserted via NdeI and BamHI sites. The final construct was verified by control digestion and sequencing. *E.coli* BL21(DE3) C41 cells harboring the pET14b[eGFP-*Sc*ASNaseI] plasmid were used for expression of the fusion protein at 22 □C in auto-induction culture medium containing lactose as inducer. The culture was inoculated with a single colony and was incubated at 22 □C for 24 h. The fusion enzyme was purified and stored as described above for the His_6-SUMO-*Sc*ASNaseI fusion.

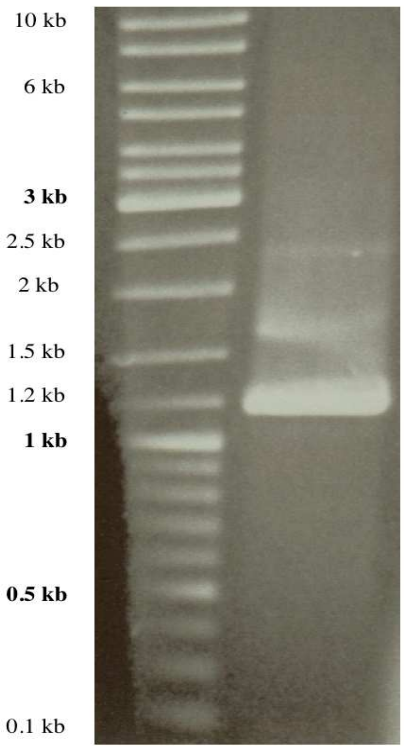

Figure S1. PCR amplification of the *Sc*ASNaseI-coding region (ASP1 gene) using as template genomic DNA from *Saccharomyces cerevisiae*. The main amplified fragment shows the expected size of 1143 bp.

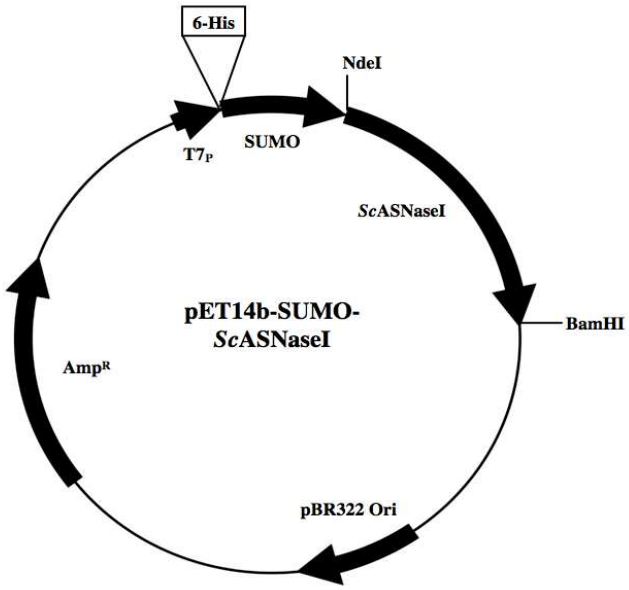

Figure S2. The final construct of the His_6-SUMO-*Sc*ASNaseI cloned into pET14b. The purified intact *Sc*ASNaseI protein (after removal of the His_6-SUMO tag by SUMO protease) is shown below in Figure S4.

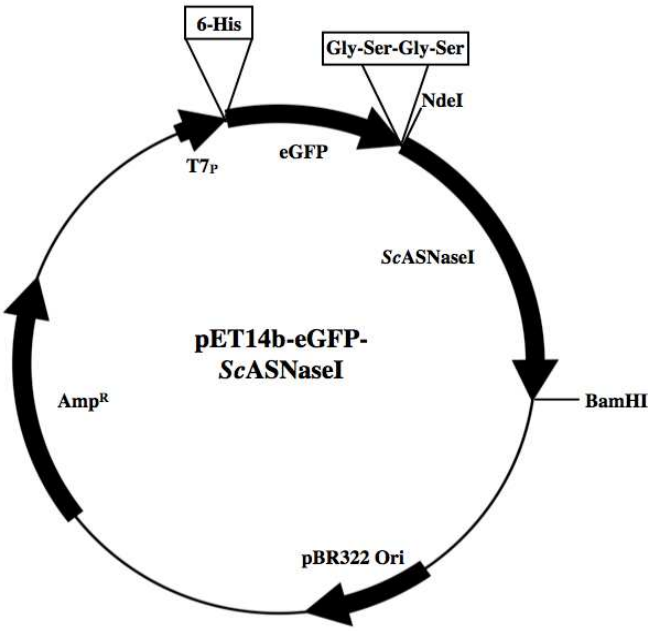

Figure S3. Construct for the expression of the His_6-eGFP-*Sc*ASNaseI fusion protein. Also indicated is the position of the short hydrophilic linker $(Gly\text{-}Ser)_2$ connecting eGFP and the enzyme.

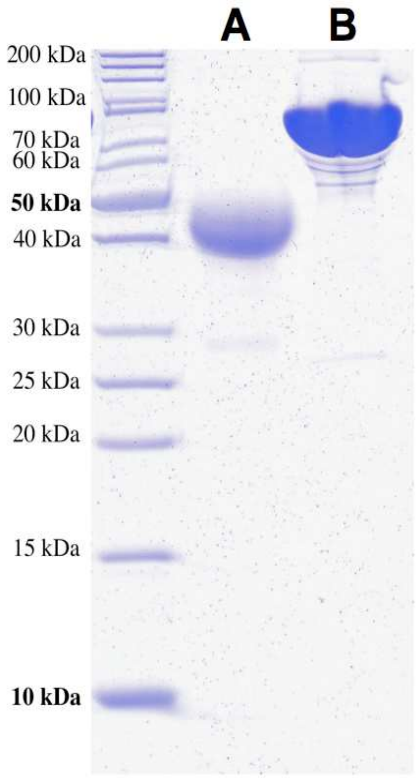

Figure S4. SDS-PAGE analysis of *Sc*ASNaseI after removal of the His_6-SUMO-tag (A), which corresponds to the expected size of ~ 40 kDa, and of the His_6-eGFP-*Sc*ASNaseI fusion (B) showing an apparent M_r ~ 70 kDa (calculated M_r: 69 kDa).

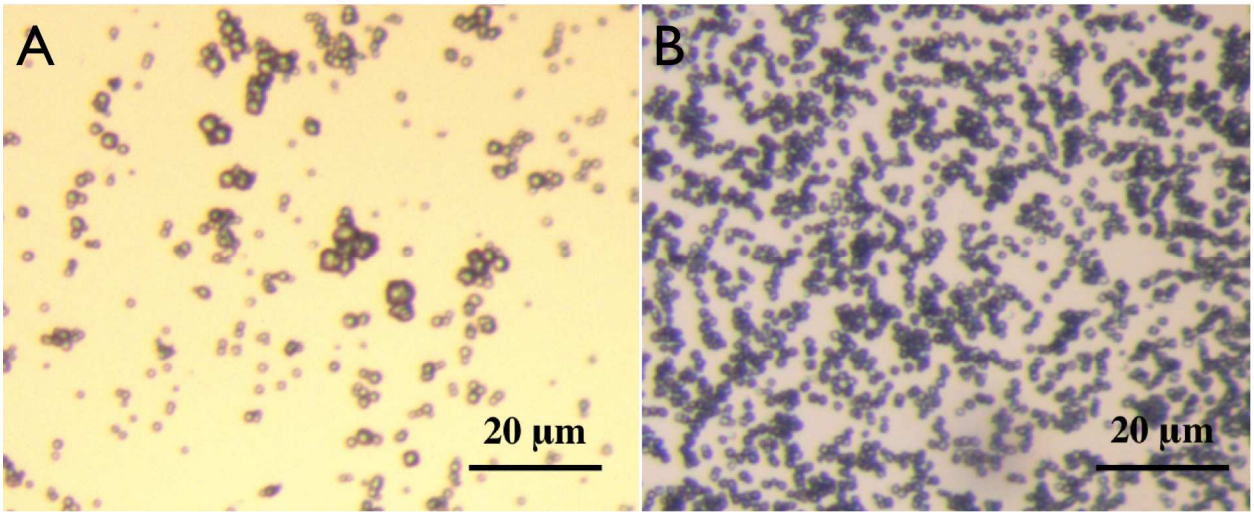

Figure S5. Protein-loaded calcium carbonate particles formed by the co-precipitation method and imaged by transmission light microscopy. Figures A and B show the L-asparaginase-filled cores, obtained when using 1 M (A) or 0.15 M (B) $Na_2CO_3/CaCl_2$.

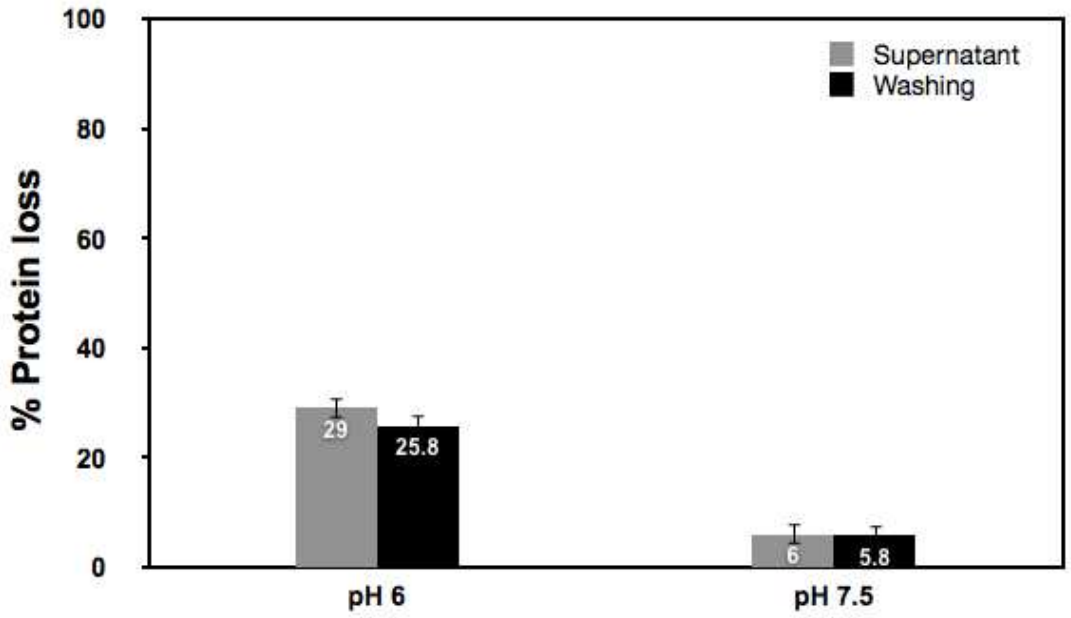

Figure S6. Comparison of the calcium carbonate/*Sc*ASNaseI loading efficiency under two pH conditions following the co-precipitation method. For quantification of protein loss, the

supernatant and the washing fractions were collected after each centrifugation step, and the total amount of protein was determined by the Bradford method. The initial amount of enzyme used was 3 mg. Results are means ± S.D. of 2 independent experiments.

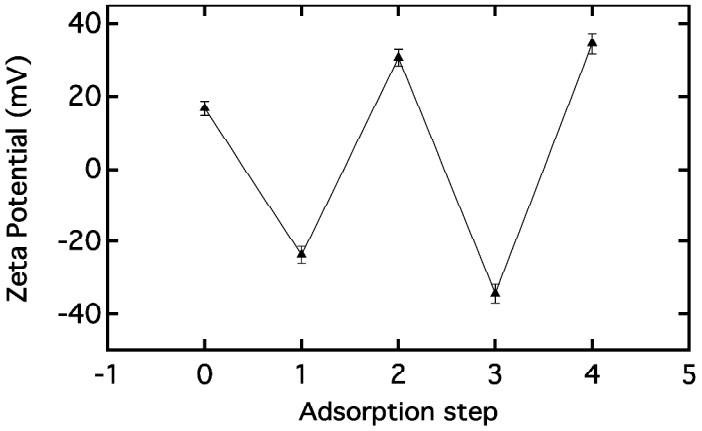

Figure S7. Zeta potential of particles determined after individual polymer adsorption steps. Step 0 defines the Z potential of the initial uncoated $CaCO_3$ particles in the presence of enzyme. Steps 1 and 3 correspond to dextran sulfate adsorption, while 2 and 4 indicate poly-L-arginine deposition. The Z potential values alternate between negative and positive upon coating with either dextran sulfate or poly-L-arginine, indicating the adsorption of poly-anions or poly-cations, respectively. Shown are the average values and the S.D. of three independent measurements.

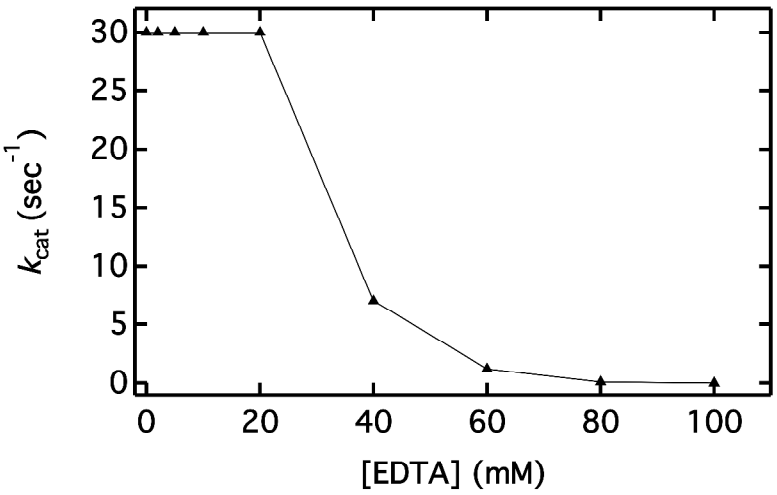

Figure S8. Inhibitory effect of EDTA on *Sc*ASNaseI activity. Measurements were performed between 0 and 100 mM EDTA using 10 µg of purified enzyme. The plot demonstrates the decrease of the steady-state turn-over rate as a function of different EDTA concentrations tested.

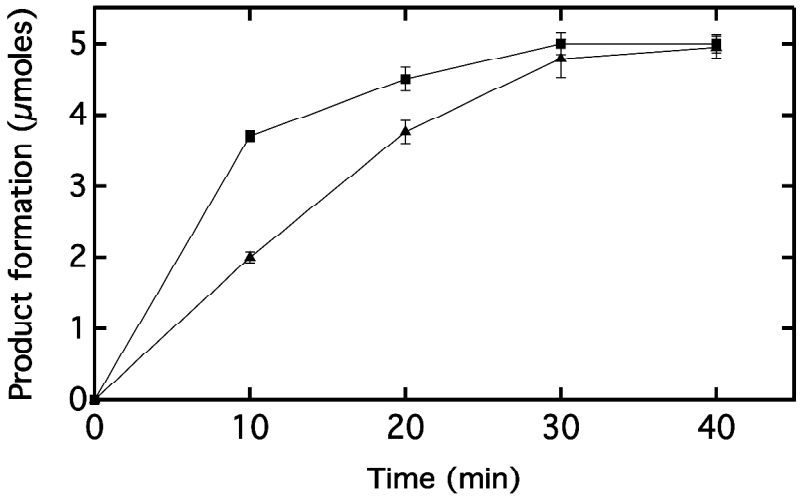

Figure S9. Time course of the enzymatic reaction when using *Sc*ASNaseI-filled capsules with either two (■) or three (▲) bilayers. The enzymatic assay was performed as described in the Methods section using L-Asn at 5 mM final concentration. For direct comparison of data, the number of capsules per volume which were used for measuring the enzymatic activity was kept

equal in both cases. Assuming that the amount of enzyme per capsule is the same in both cases, and that the loaded enzyme is equally active, the observed slower time course of the reaction may be attributed to restricted substrate, or product, diffusion in case of the 3-bilayer capsules. Shown are the average values and the S.D. of two independent experiments.

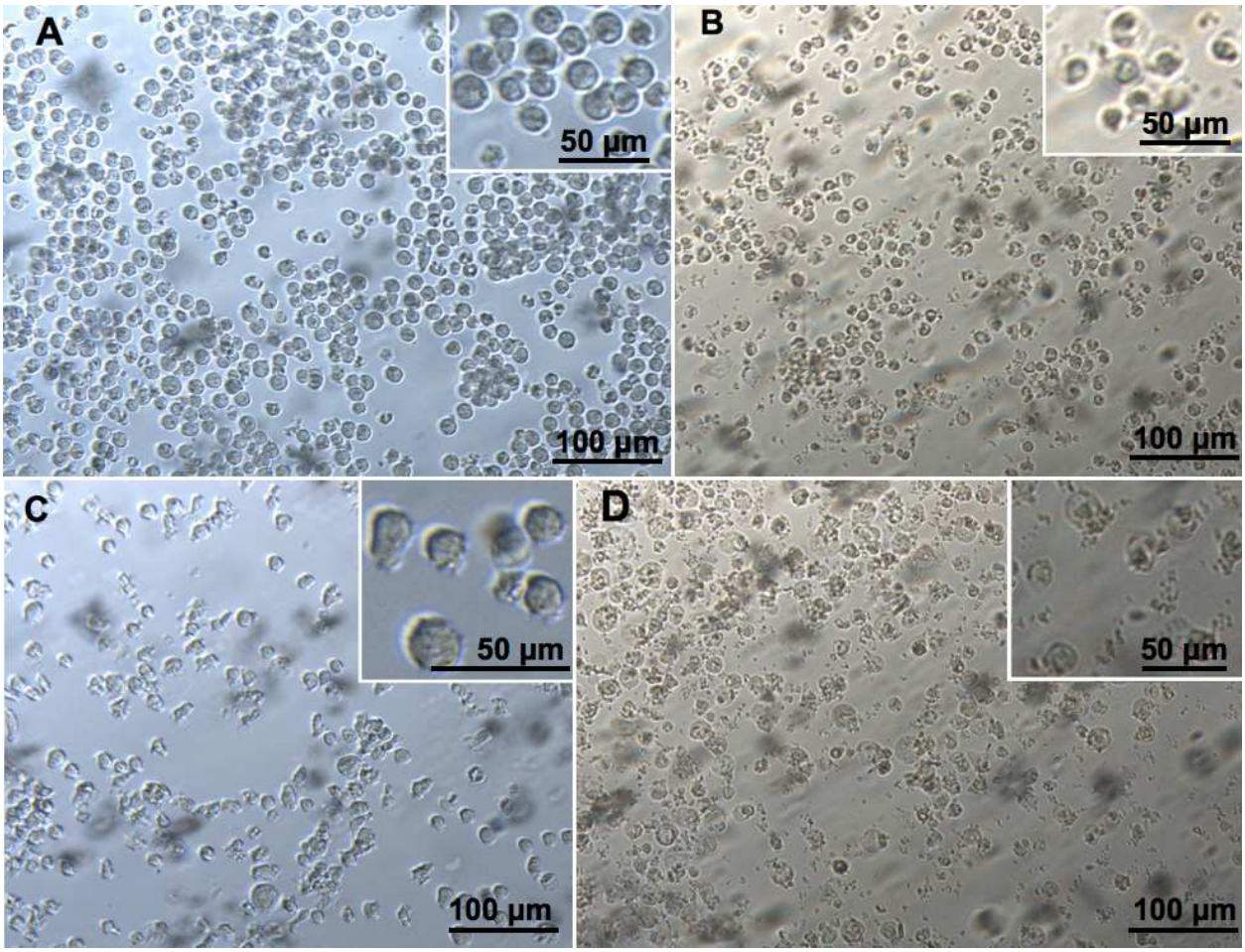

Figure S10. Transmission light microscopy images of untreated MOLT-4 (A) and SD1 (C) leukemic cells, and MOLT-4 (B) and SD1 (D) cells treated with microcapsules containing *Sc*ASNaseI. Images of the untreated cells were taken just before the drugs were added (t=0), and treated cells were imaged after 72 h of incubation, the moment at which the proliferation state was evaluated.

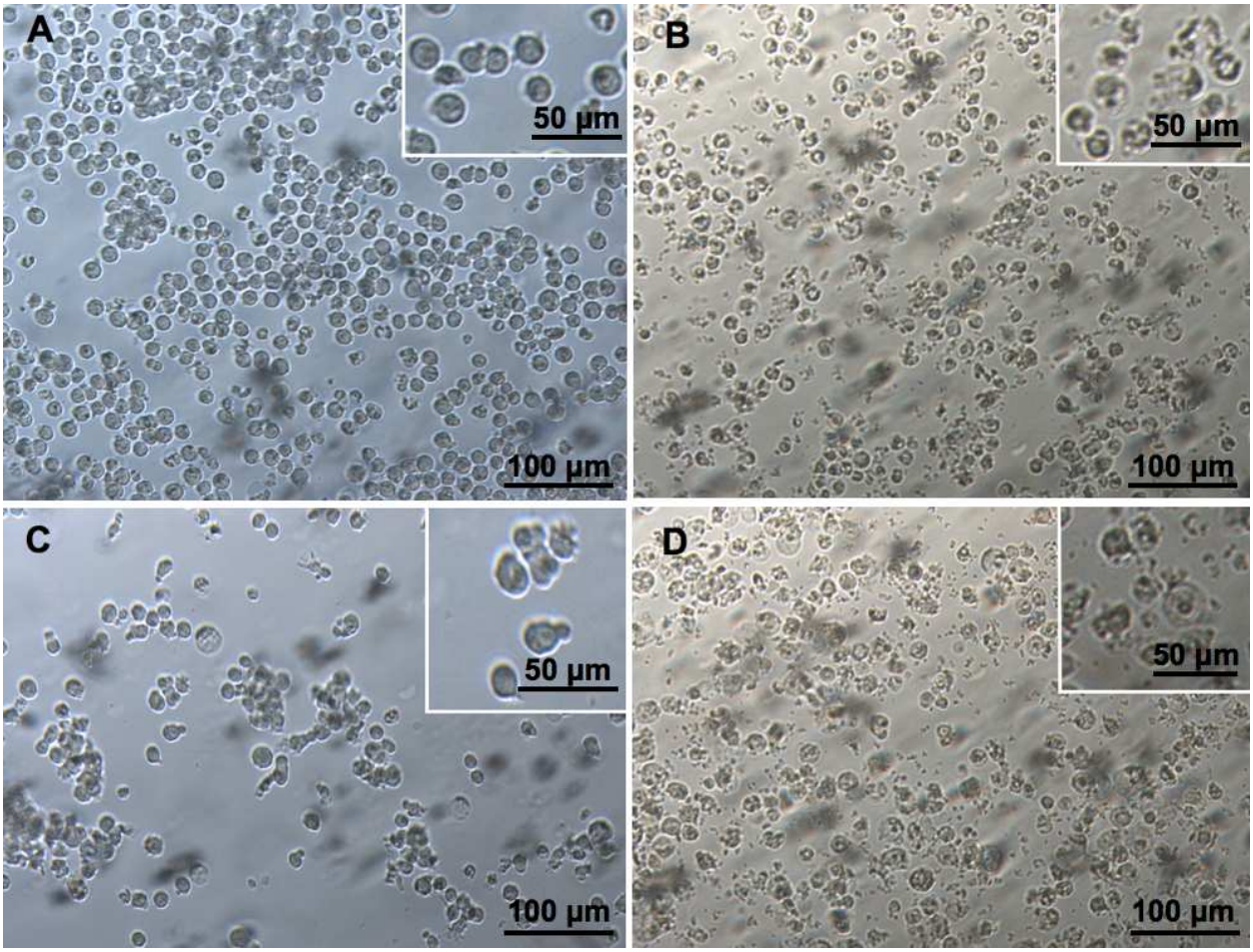

Figure S11. Transmission light microscopy images of untreated MOLT-4 (A) and SD1 (C), and of MOLT-4 (B) and SD1 (D) cells treated with *Ec*ASNaseII-filled capsules. Similarly to Figure S10, images of the untreated cells were taken just before the drugs were added (t=0), and treated cells were imaged after 72 h of incubation.