Stimuli responsive nanomaterials for therapeutic protein delivery

Farah El Mohtadi [1], Richard D'Arcy [2], Craig L Duvall [2], and Nicola Tirelli [3]

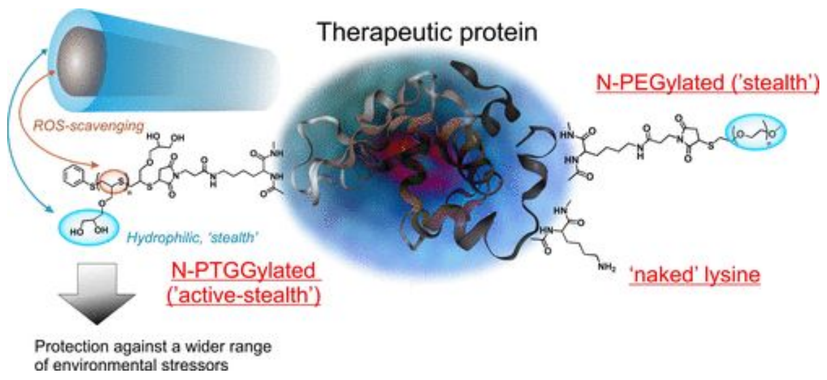
1. Portsmouth University, School of Pharmacy and Biomedical Sciences. Saint Michael's building PO1 2DT. UK
2. Arizona State University, School for Engineering of Matter, Transport and Energy, AZ85287. USA
3. Istituto Italiano di Tecnologia (IIT). Via Morego 30 16163 Genova. Italy

UNIVERSITY OF PORTSMOUTH

FACULTY OF HEALTH SCIENCES

Background

- A number of protein-based therapeutics (biologicals) have found clinical application including antibodies against specific receptors or growth factors, or actives used in e.g. enzyme replacement therapies (ERTs), or agonists/antagonists such as G-CSF (FilgrastinTM)[1].
- In order to reduce inherent immunogenicity and improve pharmacokinetics of proteins various PEG-protein conjugates have also reached clinical fruition. Their performance, however, is often hampered by inefficiencies in their production, preservation and/or storage as well as denaturation by proteolysis and oxidation[2]. There is also a growing concern over PEG immunogenicity complications like hypersensitivity reactions and advanced blood clearance (ABC)[3].
- Efforts to improve the stability of the enzyme may therefore result in considerably benefits both clinically for the patients and financially for the insurance companies/care providers. Conjugation of stimuli responsive polymers to enzymes can be used to overcome this issue.
- Anti-oxidant polymers such as polysulfides may also act to further protect enzyme activity.

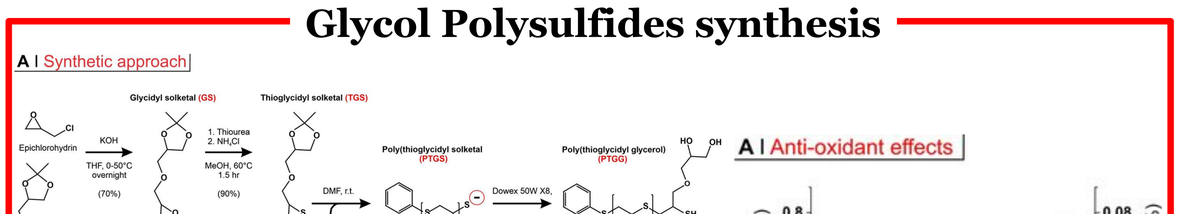


Scheme 1. PTGG (Left) Thioethers Allow for Protection against Oxidants (ROS), while Its Hydrophilic Glycerols Provide a 'Stealth' Behavior (Lower Immunogenicity, Higher Stability against Degradation, and Denaturation) Similar to or Better Than PEG (Right)

AIM

By conjugating enzymes to the synthesised polysulfide we aim to:
reduce oxidative damage thereby increasing their activity, potentially reduce/slow down proteolytic degradation, and to prevent antibodies from binding to the enzymes and thus reducing immunogenicity.

Glycol Polysulfides synthesis



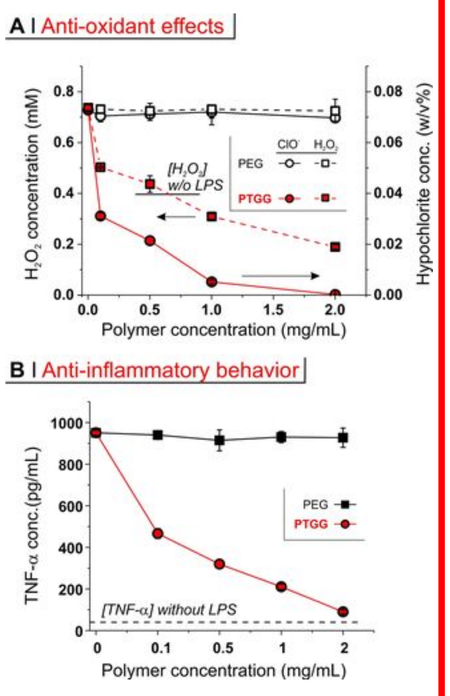


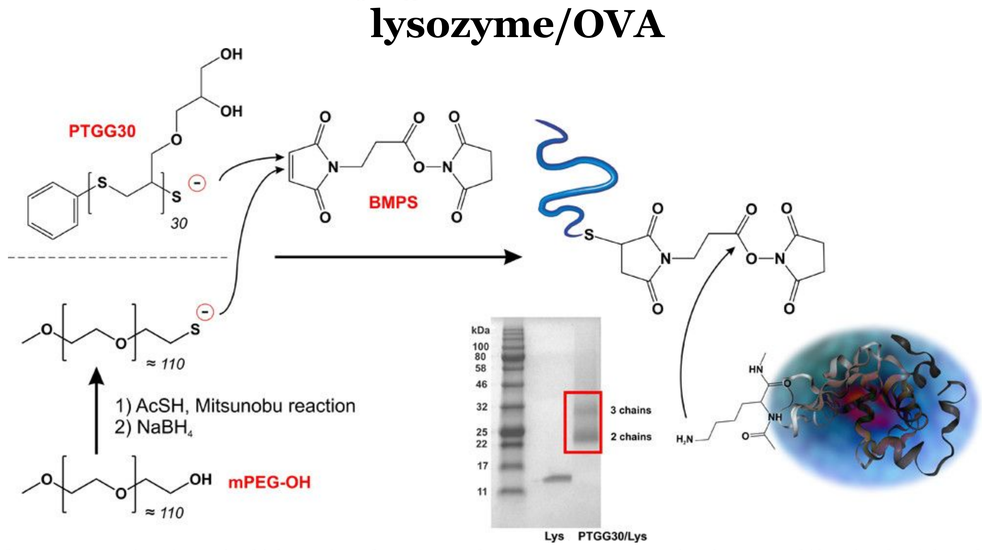
Figure 1. (**A**) TGS was produced via a two-step reaction sequence and then used in the episulfide ROP initiated by an in situ formed phenyl thiolate; the resulting PTGS was treated with an acidic resin, to deprotect glycerol side chains and protonate its terminal thiol thereby producing the final PTGG. (**B**) IR spectra of compounds prepared during PTGG synthesis (**C**) TGS consumption kinetics as determined by ^{1}H NMR in deuterated-DMF; theoretical DP = 30). In the inset, GPC traces (RI signal, triple detection in THF); the sample collected after 6 h of polymerization (PTGS30) is highlighted with an arrow; the other two refer to a 12 h of polymerization with a theoretical degree of polymerization = 60 (PTGS60) and to mPEG thioacetate; the latter and PTGS30 have very similar molecular weight distributions. (**D**) ^{1}H NMR spectra of PTGS30 in deuterated DMSO and PTGG30 in D_2O; letters correspond to the assignments on the chemical structures and shadowed areas to solvent peaks.

Figure 2. (**A**) Hydrogen peroxide (left axis, squares) and hypochlorite (right axis, circles) concentration in the cell lysates of RAW macrophages preactivated with 500 ng/mL LPS and exposed for 24 h to mPEG or PTGG30. Without LPS stimulation, hypochlorite was not detected, while H_2O_2 was present at about 0.4 mM, and it is noteworthy that high concentrations of PTGG reduced [H_2O_2] considerably below this basal level. (**B**) TNF-α concentration in RAW supernatants of preactivated RAW macrophages exposed to mPEG or PTGG30 for 24 h.

CONCLUSIONS

PTGG has demonstrated significant advantages over the current benchmark PEG and as a multifunctional, biodegradable stealth-polymer, offering an attractive alternative to PEG. The antioxidant and cryo/lyoprotective properties offer significant formulation/processing advantages to industry while the antioxidant/anti-inflammatory properties are highly attractive for e.g. anti-inflammatory protein-conjugates where a synergistic effect can be reasonably assumed, or for oxidation-sensitive proteins. PTGG therefore represents an important milestone in the advent of functional stealth polymers.

Conjugation of PTGG to lysozyme/OVA



Scheme 2. Conjugation of Thiol-Terminated PTGG and PEG to Lysozyme via the Heterobifunctional Linker β-Maleimidopropionic Acid *N*-Hydroxysuccinimide Ester (BMPS)

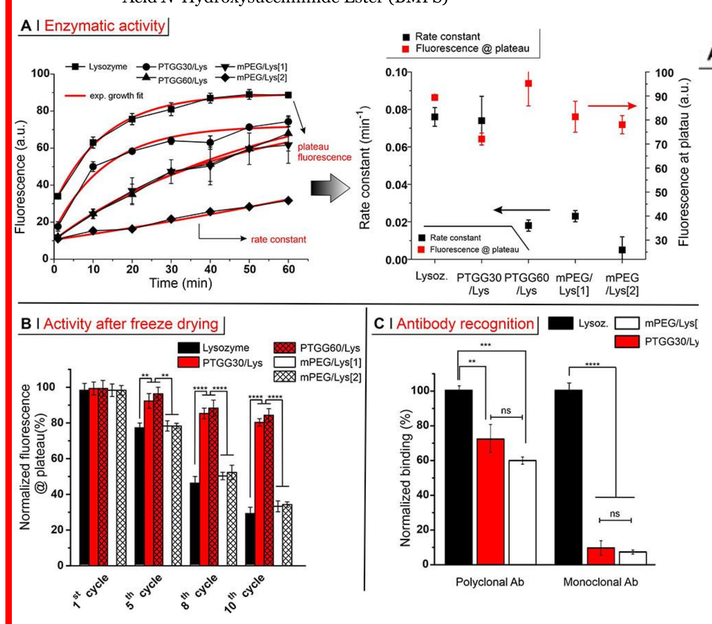


Figure 3. (**A**) Lysozyme activity was assessed using a dye-quenched assay, keeping the protein concentration constant at 0.5 mg/mL; the fluorescence was monitored over 60 min at 37 °C, fitting the data with an exponential growth equation fluorescence = A1 × exp(time/τ) + A2 (inset; red lines are fittings). A rate constant is calculated as 1/τ, while the sum A1 + A2 provides the fluorescence at plateau. (**B**) Relative activity of lysozyme and of its conjugates after 1, 5, 8, and 10 lyophilization cycles. (**C**) Binding of monoclonal or polyclonal antibodies to lysozyme derivatives was measured by direct enzyme-linked immunosorbent assay (ELISA). Statistical significance: one way ANOVA with a Tukey's means comparison; $^*P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$, $^{****}P \le 0.0001$.

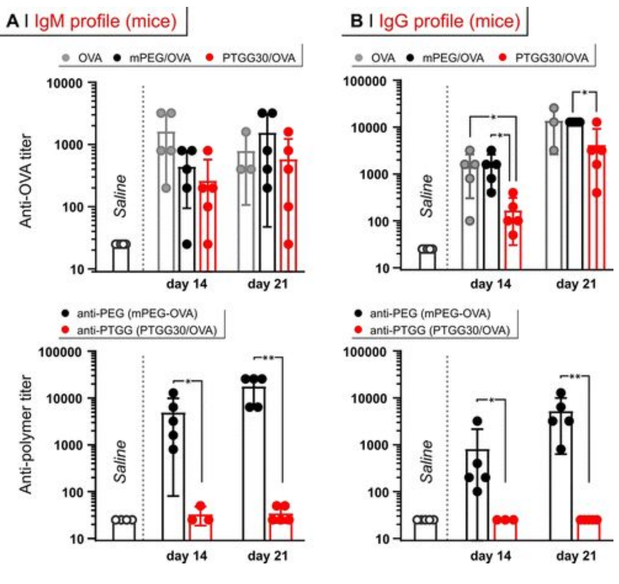


Figure 4. Immunogenicity of OVA, PEG/OVA, or PTGG/OVA was quantified via their capacity to induce the production of IgM (A) and IgG (B) against OVA (top) or the two synthetic polymers (bottom), as measured via ELISA on mice sera collected on either day 14 (animals dosed at days 1 and 7) or day 21 (dosed at days 1, 7, and 14). To test for significance, a one-way ANOVA with a Tukey's means comparison test at days 14 and 21 was performed for anti-OVA titers, and a Mann–Whitney test was used to assess antipolymer titers; $^*P \le 0.05$; $^{**}0.001 \le P \le 0.01$.

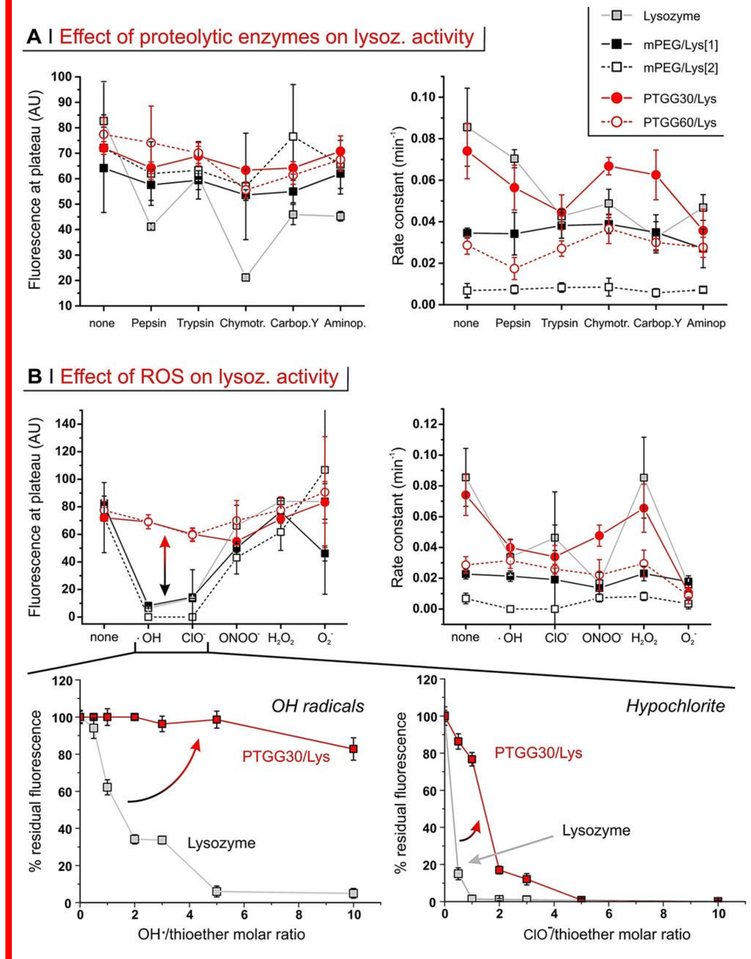


Figure 5. (**A**) Lysozyme and its derivatives (1 mg/mL) were incubated for 24 h with a panel of proteases (10 mg/mL) prior to measuring their activity and fitting their. (**B**) Top panels: activity of lysozyme and its derivatives (1 mg/mL) upon incubation with various ROS (24 h for H_2O_2 and H_2O_2/Cu(II) (OH radicals), 3 h for peroxynitrite and superoxide, and 13 min for hypochlorite; for PTGG derivatives, the oxidant/thioether molar ratios were respectively of 3:1, 5:1, 0.5:1, 3:1, and 0.5:1). Bottom panels: Normalized fluorescence at plateau upon exposure of free lysozyme and $PTGG_{30}$/Lys to increasing amounts of OH radicals (24 h) and hypochlorite (15 min); please note that the molar ratios to thioethers (horizontal scale) refer to PTGG only. (**C**) Extent of sulfide oxidation for PTGG30/Lys upon 24 h incubation with various ROS at 10:1 oxidant/thioether molar ratio (left)

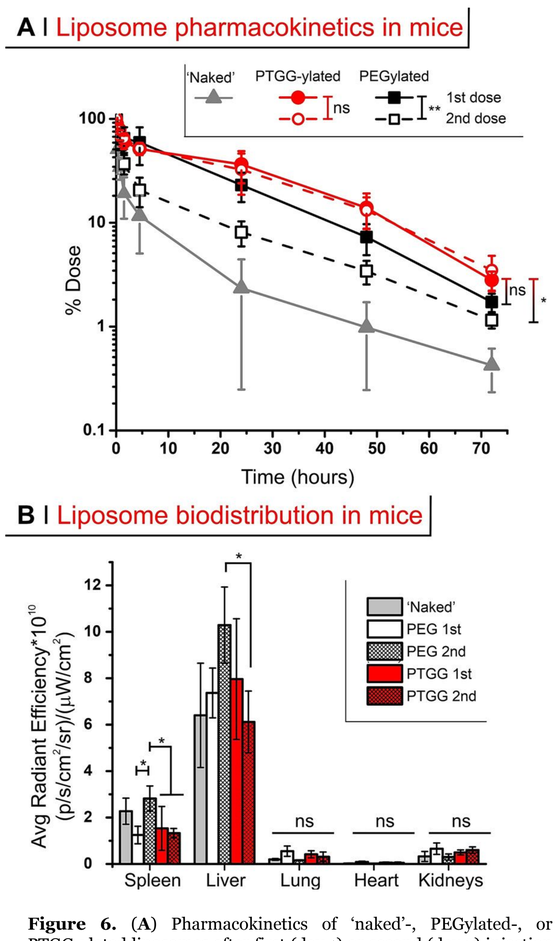
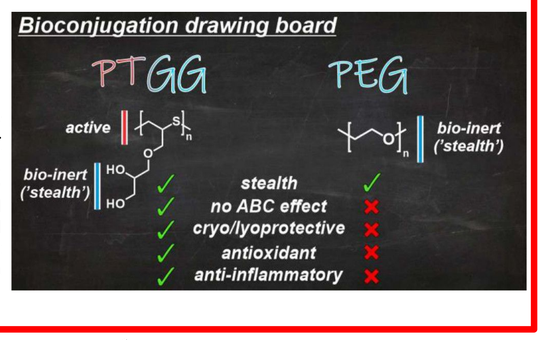


Figure 6. (**A**) Pharmacokinetics of 'naked'-, PEGylated-, or PTGG-ylated liposomes after first (day 1) or second (day 7) injection. (**B**) Biodistribution of the liposomes as assessed by measuring the fluorescence of DiD in the different organs (see Supporting Information, Figure S9). A two-way ANOVA was used to assess statistical significance within treatment groups at different doses (legend) whereas a one-way ANOVA with a Tukey's means comparison test was used to assess statistical significance between groups at 72 h; $^*P \le 0.05$; $^{**}0.001 \le P \le 0.01$.



Email: farah.elmohtadi@port.ac.uk

1. Park E et al. *Expert Opin. Emerg. Drugs* 2019, 24, 107–119 **2.** Chang C et al . *Nat. Commun.* 2017, 8, 522. 3. Heo, Y et al. *Drugs* 2019, 79, 767–777.