Incorporation of encapsulated cytokines into tissue scaffold provides stabilization and protection during processing and storage

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Statement of Purpose: It is likely that tissue scaffolds for use in tissue engineering and regenerative medicine will need to serve as a depot for release of labile protein cytokines that will guide cell function in a desired way. tissue scaffolds is likely to be critical to making functional scaffold of close mimic of natural extracelluar matrix (ECM) for tissue engineering & regenerative medicine (TERM). These cytokines are often very potent factors for guiding cell differentiation and function, but they are also typically quite labile. The present strategies for loading cytokines into scaffolds consist of surface immobilization or bulk addition of protein as water emulsion or freeze-dried powder to a solvent-based polymer solution and subsequent casting of a tissue scaffold from the protein-bearing solution. While a number of reports using this approach have been published, we believe that it is simply not tenable for at least two reasons. The first, and most compelling, is that proteins incorporated into scaffolds in this manner are subject to significant chemical and physical degradation during processing. As a consequence of this high degradation rate, large amounts of protein must be incorporated in order to deliver even a very small amount of functional protein (see Figure 1) . Aside from being wasteful of precious cytokines, this approach poses significant safety risks, and would not likely be acceptable to the FDA. The large amounts of damaged protein has a significant probability of being immunogenic. Furthermore, proteins incorporated to scaffolds using bulk addition are likely to have poor storage stability, leading to increased degradation over time in addition to damage incurred during processing. Methods used to incorporate cytokines into tissue scaffolds will need to protect the proteins from process and storage degradation, even at very low (e.g., nanomolar) loading levels.

Methods: We have encapsulated several proteins - horseradish peroxidase (HRP), candida rugosa lipase, insulin and BMP2 - separately in surfactant-capped sugar-glass nanoparticles of diameter (20 to 50) nm. Encapsulation is achieved by flash-freezing an inverse micelle suspension in which the aqueous phase contains protein and sugar. After flash-freezing, the suspension is freeze-dried, leaving a small number of protein molecules embedded in each nanoparticle. The protein-bearing nanoparticles are then suspended in an organic solvent-based polymer solution, from which a tissue scaffold is formed by a process such as electrospinning, or gas foaming. We have tested the protein in nanoparticles for resilience to processing, and for amount of active protein delivered from the scaffolds. Resilience to processing was tested by exposing the protected and unprotected protein to various organic solvents commonly used in scaffold preparation(e.g. dichloromethane, chloroform, toluene, cyclohexane, isooctane, tetrahydrofuran, methanol, acetone). Protein activity was measured after incubation; reaction catalytic activity of HRP and lipase, and *in vitro* biological activity of insulin using L6 rat myoblast cells.

Results: The proteins encapsulated in sugar nanoparticles are protected from harsh processing conditions, including exposure to organic solvent. Protected protein retains at least 90% of its catalytic and biological activity after 2 h exposure to any of the solvents tested except methanol, and more than 70% of its activity after 24 h exposure. On the other hand, no measurable protein activity is retained when unprotected protein is exposed to any of these solvents, even at 2 h. Exposure to methanol resulted in 80 percent loss of protein activity for both the protected and unprotected protein. Figure 1 shows HRP activity after mixing protected and unprotected protein into 30 mL of 100 mg/mL poly(e-caprolactone) in chloroform for .5 h. Protected protein retained essentially 100 % of its activity, whereas all measurable activity is lost for unprotected HRP when less than 1 pmole protein is added

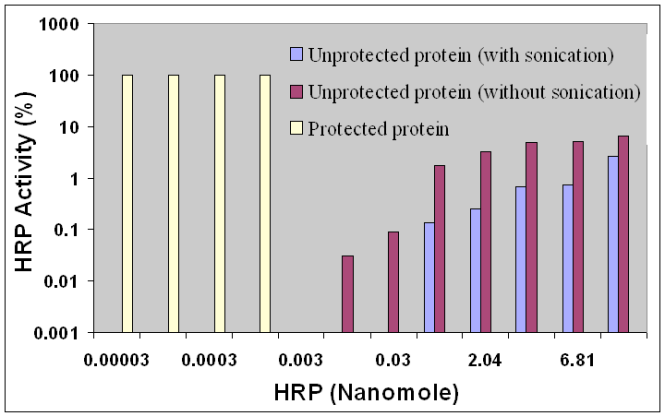

Figure 1 HRP activity after 0.5 h exposure to 10% PLCL in chloroform. Sonication of protected and unprotected protein was carried out for 30 s. Uncertainty was +/- 3% of measured values.

to the solution. Activity retention gradually increase with increase the HRP concentration (up to 14 nmole), but in no case exceeded 7 %. Release studies show that the protected HRP slowly eluted (over 30 days) as the scaffold degrades in an aqueous environment.

Conclusions: We have demonstrated a method for incorporating proteins into tissue scaffolds with essentially no loss of activity at arbitrarily low levels of protein addition. The sugar nanoparticles used to protect proteins provide excellent dispersion in the polymer matrix and stability to the protein cytokines during scaffold processing as well as storage. Slow, quantitative release of bioactive cytokine provides promise for using this approach to develop functional scaffolds for complex tissue regeneration.