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Entwicklung von reagenzfrei kovalentes Quervernetzen der chitosan-gelatine Grundgerüste
Development of reagent-free covalent crosslinking of chitosan-gelatin scaffolds

Introduction

Scaffolds based on composite chitosan-gelatin materials show promise in tissue engineering and drug delivery for reasons related to biocompatibility, biodegradability, inherent biocidal activity, and easy preparation in diverse forms, with variable mechanical properties, porosities and interconnectivities [1, 2]. Normally regarded as a soft polymer, the incorporation of such formulations in mechanically challenging applications would necessitate the use of established crosslinking agents such as formaldehyde and glutaraldehyde, amongst others [3, 4]. While effective, reagent use carries along concerns in view of the direct toxicity of many crosslinkers as well as the potential or established threat posed during *in vivo* degradation by their breakdown products. Zero-length crosslinkers such as carbodiimides, which incorporate no foreign structure into the biomaterial, have partially alleviated such concerns, but nonetheless the risk of residual reagent has still remained a subject of concern [5]. Most recently, the use of naturally available and relatively non-toxic crosslinkers such as genipin has impacted biomaterials research [6]. An excellent cross-linker of proteins, collagen, gelatin, chitosan and combinations thereof, the low acute toxicity of genipin potentially forecasts major changes to materials that are normally prepared using synthetic cross-linking regents.

In keeping with the above discussion, another ideal crosslinking strategy could conceivably follow a reagent-free approach, as might be described by the thermally-promoted transformation of ammonium carboxylate bridges into covalent amide bonds. Such pyrolysis reactions occur when carboxylic acids and primary (or secondary) amines are reacted to yield salts, which are subsequently incubated (200˚C) to evolve water [7]:

$$RCOOH + H_2N\text{-}R' \rightarrow RCOO^{-}\,{}^{+}NH_3\text{-}R' \rightarrow RCONH\text{-}R' + H_2O_{(g)}$$

A general method has already been established to desolubilize protein powders by intermolecularly cross-linking bridged ammonium carboxylate groups [8]. This mechanism has been confirmed directly in later work [9]. In these protein-based materials, covalent bonds were formed at relatively low temperatures (i.e., 75-100ºC) by applying a vacuum to better drive off water. Herein, the same *in vacuo* principle has been extended to crosslink chitosan-gelatin composites:

$$\text{Gelatin-}COO^{-}\,{}^{+}NH_3\text{-Chitosan} \rightarrow \text{Gelatin-CONH-Chitosan} + H_2O_{(g)}$$
$$\text{Gelatin-}COO^{-}\,{}^{+}NH_3\text{-Gelatin} \rightarrow \text{Gelatin-CONH-Gelatin} + H_2O_{(g)}$$

It is hoped that this preliminary work may highlight the merit of *in vacuo* thermal treatments as an alternative to crosslink biomaterials and to effect changes of solubility, degradability and mechanical properties.

Materials and Methods

Stock solutions: Chitosan was dissolved in acetic acid (1wt%) under vigorous agitation. NaOH (1M) was added dropwise until a pH of 6 was established. The volume was adjusted to yield a prescribed chitosan concentration (2-4wt%). Finally, gelatin (2-8wt%) was dissolved with mild stirring and the pH was readjusted as required.

Lyophilisate-type scaffolds: Stock solutions (1ml or 2ml) were flash-frozen in dry ice-ethanol and lyophilized without external cooling (0.02-0.04mbar, 36h).

Aggregate-type scaffolds: Stock solutions (0.1ml) were added rapidly into tubes containing nine volumes ethanol under vigorous agitation (1 minute) to afford a white precipitate. The tubes were centrifuged, the supernatant was discarded, and the precipitates were air-dried.

In vacuo thermal crosslinking: A make-shift apparatus comprising a block heater or temperature-regulated oil bath and vacuum line (figure 1) was employed to drive the *in vacuo* crosslinking (100ºC, 0.2-0.6mbar, 24h).

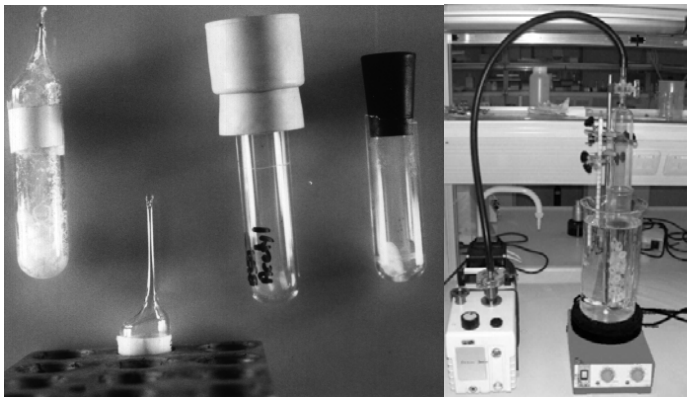

Figure 1. Setup to crosslink protein samples individually (left) or collectively (right) under vacuum conditions and elevated temperatures.

Solubility tests: Lyophilized/lyophilized-heated samples were dispersed and agitated in PBS solution (phosphate buffered saline pH 7.4, 1wt% protein, 37˚C). Aliquots of supernatant were removed over time and assessed for amino group content using the ninhydrin reagent [10].

SEM analyses: Samples were frozen in liquid nitrogen and cut with a fresh razor blade before treatment with a carbon coater. A gun voltage of 5-10kV was employed.

Results

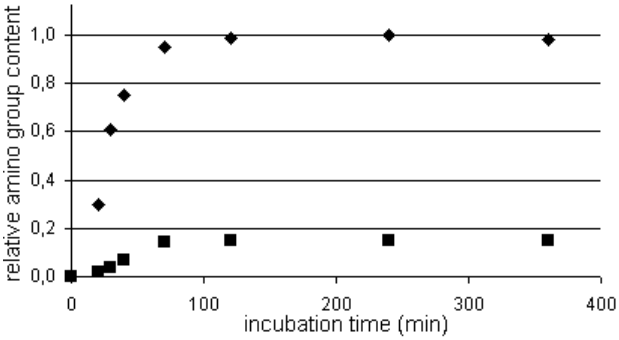

Figure 2. Relative dissolution of co-lyophilized chitosan-gelatin (2wt%: 2wt% originally) when reconstituted in PBS medium before (♦) and after (■) thermal treatment.

The thermal treatment of co-lyophilized chitosan-gelatin yielded materials with markedly reduced solubility as exemplified by the analysis in figure 2. Unfortunately, the thermal treatment of co-lyophilisates was accompanied by a degradation of the original pore structure (figure 3a versus 3b). Comparatively speaking, thermally treated aggregates (figure 3c) exhibited even less porosity. Unheated aggregates were also nonporous.

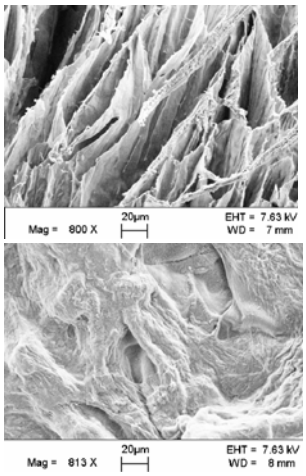

Figure 3. Morphology of chitosan-gelatin (2wt%: 2wt%) colyophilisate (top, left); *in vacuo* thermalized colyophilisate (top, right); and *in vacuo* thermalized aggregate (bottom, left).

Discussion

This preliminary study examined the feasibility, technical challenges and potential merit of employing an *in vacuo* condensation-based crosslinking strategy to fabricate low-to-zero toxicity scaffolds, with advantages presumed in application and conveniences in legislation.

SDS PAGE analyses of *in vacuo* treated albumins (results not shown) had indicated high crosslinking, as most of the material could not enter the gel matrix. Since these samples had been boiled in the presence of mercaptans, the potential modes of crosslinking clearly excluded a disulfide contribution. Other studies on proteins had established the role of amide bonding, implying at least a partial contribution, and had hinted to the possibility of inter-protein ester bonding [8, 9]. In reviewing the structure of chitosan, *in vacuo* thermally-induced amidation (and possibly esterification) described the only mode(s) of covalent crosslinking to gelatin.

Unlike other typical biological materials, a dry powder consisting of chitosan and gelatin could not be directly manipulated at pH values nearing neutrality. The stocks were necessarily prepared in a stepwise fashion at lower pH values and later brought to pH 6. As such, the protocol was comparatively tedious with respect to other protein preparations such as albumin [9]. The limited solubility of chitosan was a factor, as was the high viscosity imparted by gelatin, which made stirring, pH adjustments, and flash freezing of pre-lyophilisates or rapid precipitation of aggregates very difficult.

Irrespective of certain difficulties, the overall findings were not discouraging. Thermally treated samples remained insoluble even after a one-month incubation in PBS buffer, whereas unheated lyophilisates and aggregates had all but dissolved within a few hours. Even neat formic acid failed to dissolve thermally treated samples over a period of hours. While the structural densification of lyophilized chitosan-gelatin was an undesired finding of the *in vacuo* thermal treatment, the result nonetheless indicated that interactions within the matrix had changed considerably over the course of heating. Indeed, minor shrinkage of the heated lyophilisates was even apparent to the naked eye. In addition to demonstrating insolubility following thermal treatment, the chitosan-gelatin matrix also proved mechanically robust, as these samples were much more difficult to cut, compress and otherwise fragment.

Summary

While this initial study clearly highlighted a potential for future development, a serious drawback over established methods was the loss of porosity and interconnectivity. Studies are currently underway to ascertain the best conditions to maintain or potentiate matrix porosity via inclusion of non-crosslinkable or thermally-activated gas-phase porogens [11]. Once these conditions are established, what remains is to unequivocally confirm the nature of the crosslinking, the degree of crosslinking, the degradation time, and the stress-strain behavior.

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