A specific quantitative assay for collagen synthesis by cells seeded in collagen-based biomaterials using sirius red F3B precipitation

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The measurement of collagen synthesis by seeded cells *in vitro* is a prerequisite for the assessment of biocompatibility of many biomaterials. Existing methods are either complicated or not applicable to systems utilizing collagen-based materials, and the development of a rapid and simple technique would be an advantage. In the current paper, a method is described which relies on the radiolabelling of newly synthesized protein with [^{3}H]-proline followed by specific precipitation of collagen using 1% sirius red dissolved in water. The results indicate that collagen binding to sirius red is unaffected by using water rather than picric acid as a solvent and the dye binds in a similar fashion to collagen type I, II and III. Cycloheximide treatment of the gels indicated that precipitated [^{3}H]-proline was restricted to macromolecular protein. Collagenase treatment eradicated labelled precipitation formation when using 1% sirius red in water, indicating a high degree of specificity for collagen whilst specificity was poor when using 1% sirius red in picric acid. The method described is both simple and rapid and shows a high degree of specificity and sensitivity. For these reasons it is highly suited for the assessment of collagen synthesis by cells in collagen-based materials.

1. Introduction

In recent years there has been a growing interest in the use of collagen and collagen-based biomaterials in the manufacture of medical devices and repair systems [1, 2]. Of particular interest is the proposed use of resorbable collagen-based systems, seeded with cells for tissue-engineered repair systems [3, 4]. In many cases, the ability of the cells to synthesize collagen within the system is crucial to the overall success of the system. It is, therefore, important to assess collagen synthesis by cells within collagen-based systems *in vitro* in order to determine the biocompatibility and bioactivity of the device. Measurement of the rate of collagen synthesis within collagen-based systems is, however, problematic. Methods which assess total collagen are not appropriate for collagen gels, due to levels of pre-existing collagen [5], whilst methods which detect procollagen fragments suffer from entrapment phenomena within the gel, cannot differentiate between fibrillar and degraded collagen, and are specific for only one collagen type [6, 7]. The use of radiolabelled proline incorporation provides an alternative approach but must be made specific for collagen rather than total protein. Chromatography may be used to determine radiolabelled-hydroxyproline in proline-labelled cultures [8, 9]. Alternatively, radiolabelled protein may be precipitated with subsequent collagenase treatment to determine specific incorporation [10]. Both methods are complicated and fraught with potential pit-falls. The use of an agent which will precipitate collagen alone would overcome these problems.

This study reports the development of a system based on the specific precipitation of collagen by sirius red F3B, which binds to the [Gly-x-y] helical structure found in all collagens [11–14]. The elongated sirius red molecule binds to triple helical collagen molecules in a parallel fashion and therefore will not bind to denatured or degraded collagen or to other proteins which do not possess the typical collagenous triple helical structure. The dye has been extensively used as a histological stain for collagen and for the quantification of total collagen content. In the aforementioned applications, the dye has been dissolved in picric acid which exhibits non-specific precipitation of proteins, although only collagen is stained [14]. This is not appropriate for the assessment of collagen synthesis using radiolabelled proline incorporation, as labelled non-collagenous proteins may be precipitated. In this study we report a modification in which the dye is dissolved in water to increase specificity of precipitation for collagen. Method validation studies were performed using dermal fibroblasts cultured within collagen gels. A subsequent time course study,

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to determine variations in collagen synthesis rate with time in culture, was performed using the method characterized in this study.

2. Materials and methods

2.1. Sirius red dye preparation

Sirius red dye solutions were prepared by dissolving 1 g sirius red F3B (Raymond A. Lamb, London, UK) in 100 ml saturated aqueous picric acid (HD supplies, Hemel Hempstead, UK) or distilled water. Both dye solutions were supplemented with 0.02% Tween 20 (BDH, Poole, UK).

2.2. Sirius red dye binding to collagen

Solutions of collagen types I (Sigma, Poole, UK), II (gift from Professor Archer, Cardiff) and III (Sigma, Poole, UK) were prepared in 0.5 M acetic acid. To determine dye binding to collagen, 250 µl dye solution (1% sirius red in picric acid or distilled water) was added to the wells of a 96 well Multiscreen® filtration plate (0.65 µm pore size Millipore, Watford, UK) followed by 50 µl collagen solution at concentrations ranging from 0–1000 $\mu g\,ml^{-1}$ in 0.5 M acetic acid. The plate was sealed and agitated at room temperature for 30 min. The solution was vacuum aspirated and the precipitate was washed three times in 200 µl 0.05 M acetic acid. The precipitate was resuspended in 250 µl 0.5 M NaOH for 30 min with agitation. Aliquots of the solution were transferred to a 96 well plate and the absorbance at 540 nm determined using a BioRad 3550 microplate reader (BioRad, Hemel Hempstead, UK).

2.3. Culture of cells in collagen gels

Fibroblast-seeded collagen gels were prepared by mixing 12 ml double strength Dulbeccos Modified Eagle Medium (DMEM, Gibco, Paisley, UK), 3 ml cell suspension (1×10^6 cell ml^{-1} in DMEM), 3 ml foetal calf serum (FCS, Gibco, Paisley, UK), 3 ml 0.1 M NaOH and 9 ml collagen solution (3 $mg\,ml^{-1}$ collagen type I in 0.5 M acetic acid, ICN, Paisley, UK). All components were maintained at 4 °C during mixing. Aliquots (1.5 ml) of the solution were added to wells and gelled at 37 °C/5% CO_2 for 30 min. The gels were maintained at 37 °C/5% CO_2 in DMEM + 10% FCS + 150 $\mu g\,ml^{-1}$ ascorbate with medium changes every 2 d for 6 d to allow maximal multiaxial contraction [3]. The gels were subsequently incubated in DMEM + 10% FCS supplemented with 150 $\mu g\,ml^{-1}$ ascorbate and 10 $\mu Ci\,ml^{-1}$ [^{3}H]-proline (Amersham International, Amersham, UK) for 24 h. Some samples were additionally supplemented with 20 $\mu g\,ml^{-1}$ cycloheximide (Sigma, Poole, UK) during the labelling period. At the end of the labelling period, the gels were removed from culture, weighed, lyophilized, weighed again and digested with 1 $mg\,ml^{-1}$ pepsin (Sigma, Poole, UK) in 0.5 M acetic acid at 4 °C overnight.

In a separate experiment, gels were prepared and labelled as before. At the end of the labelling period, the gels were weighed, lyophilized, weighed again and digested in 1 ml PBS supplemented with 100 unit ml^{-1} collagenase (CLSPA, Worthington, Reading, UK) for 2 h at 37 °C. At the end of the collagenase digestion the gels were lyophilized and digested with 1 $mg\,ml^{-1}$ pepsin (Sigma, Poole, UK) in 0.5 M acetic acid at 4 °C overnight. Controls were incubated for 2 h at 37 °C in PBS without collagenase.

In a further experiment, gels were prepared as before and labelled with 10 $\mu Ci\,ml^{-1}$ [^{3}H]-proline on day 1, day 3, and day 6 of culture. At the end of the labelling period, the gels were removed from culture, weighed, lyophilized, weighed again and digested with 1 $mg\,ml^{-1}$ pepsin (Sigma, Poole, UK) in 0.5 M acetic acid at 4 °C overnight.

2.4. Determination of the rate of collagen synthesis

To precipitate radiolabelled collagen, 250 µl of the dye solution (1% sirius red in picric acid or distilled water) was added to the wells of a 96 well Multiscreen® filtration plate (0.65 µm pore size, Millipore, Watford, UK) followed by 50 µl pepsin digested sample 0.5 M acetic acid. The plate was sealed and agitated at room temperature for 30 min. The solution was vacuum aspirated and the precipitate was washed three times in 200 µl of 0.05 M acetic acid. The filters were dried and punched out into scintillation vials using a Multiscreen® 8-up punch (Millipore, Watford, UK). The filters were agitated for 30 min in 0.5 ml 0.5 M NaOH to resolubilize precipitated [^{3}H]-proline into solution and counted in 4 ml scintillation fluid (Emulsifier Safe, Packard, Pangbourne, UK) using a Tricarb 4000 series counter (Packard, Pangbourne, UK). To determine the specificity of the dye, assays were performed using 0.02% Tween 20 in saturated aqueous picric acid or distilled water in place of the dye solutions. For comparison, [^{3}H]-proline incorporated into protein was assessed by 10% TCA precipitation using the Millipore Multiscreen® system. Total DNA was measured using the Hoescht 33258 method [15].

3. Results

3.1. Sirius red dye binding to collagen

The binding of various collagen types to sirius red in picric acid or water, measured as absorbance at 540 nm, is presented in Fig. 1a (type I collagen), 1b (type II collagen) and 1c (type III collagen). No significant differences in the binding profile of individual collagen types between the two dye solutions was detected. Slight differences in the absolute absorbance levels were observed between collagen types, although all exhibited a linear relationship between absorbance and collagen concentration at concentrations up to 1 $mg\,ml^{-1}$.

3.2. Determination of the rate of collagen synthesis

The incorporation of [^{3}H]-proline into proteinaceous material, measured by precipitation using sirius red in

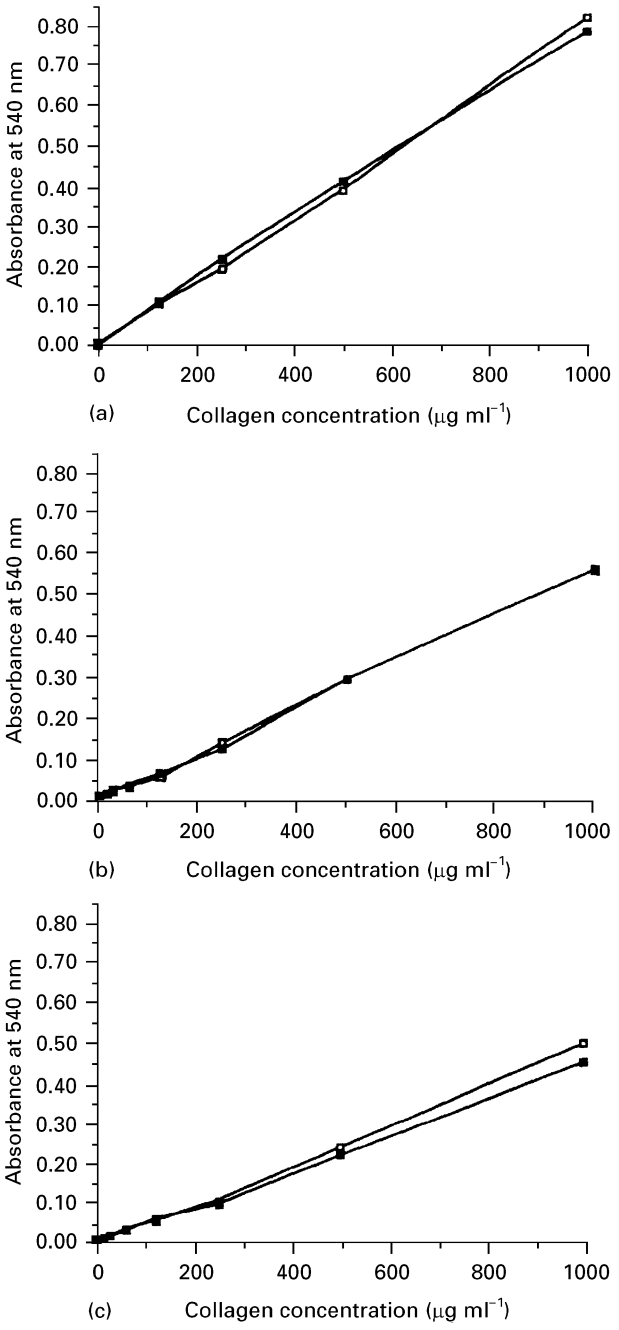

Figure 1 Binding of collagen (a) type I, (b) type II and (c) type III solutions in 0.5 M acetic acid to 1% sirius red F3B in either (□) saturated aqueous picric acid or (■) distilled water, determined by absorbance at 540 nm. Each point represents the mean of four replicates.

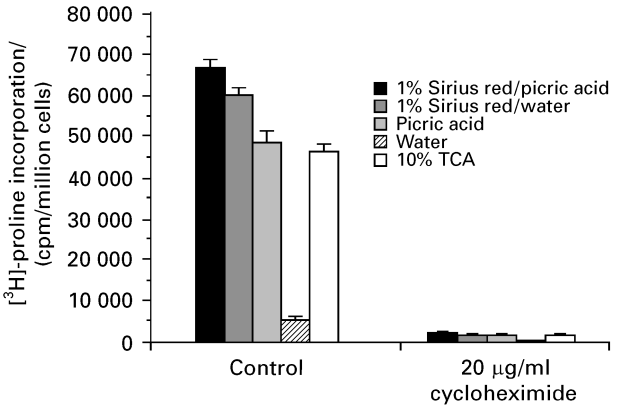

Figure 2 Tritiated proline incorporation by fibroblasts within collagen gels with or without cycloheximide, measured by precipitation of pepsin digested samples with or without 1% sirius red in either saturated aqueous picric acid or distilled water or 10% TCA. Each point represents the mean and standard error of four replicates.

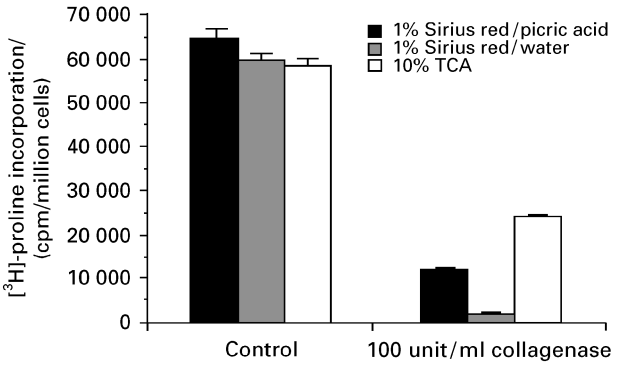

Figure 3 Tritiated proline incorporation by fibroblasts within collagen gels with or without collagenase treatment, measured by precipitation of pepsin digested samples with 1% sirius red in either saturated aqueous picric acid or distilled water or 10% TCA. Each point represents the mean and standard error of four replicates.

either picric acid or water and by 10% TCA is illustrated in Fig. 2 for both control and cycloheximide treated samples. The amount of labelled material precipitated by picric acid alone was 73% of the level measured using 1% sirius red in picric acid. In contrast, precipitation by water alone was only 9% of the level measured using 1% sirius red in water. Cycloheximide treatment, which inhibits protein synthesis, reduced incorporation by approximately 97% in all cases.

Collagenase treatment of the labelled gels reduced precipitation of incorporated [^{3}H]-proline by 81%, 96% and 57% for 1% sirius red in picric acid, 1% sirius red in water and 10% TCA respectively (Fig. 3).

The incoporation of [^{3}H]-proline into collagen by fibroblasts seeded in collagen gels and labelled on day 1, day 3 and day 6 of culture is illustrated in Fig. 4. The results revealed a decline in the rate of collagen synthesis with time in culture, such that the rate on day 6 was 44.5% of the rate on day 1.

4. Discussion

The measurement of collagen synthesis by cells seeded within collagen-based biomaterials is important for the assessment of the biocompatibility and bioactivity of the materials. The object of the present study was to develop a method for measuring collagen synthesis based on the selective precipitation of [^{3}H]-proline labelled collagen which overcomes the problems associated with other methods. In order for collagen synthesis to be measured effectively, however, precipitation must be restricted to macromolecular collagen and not to other proteins or to free proline.

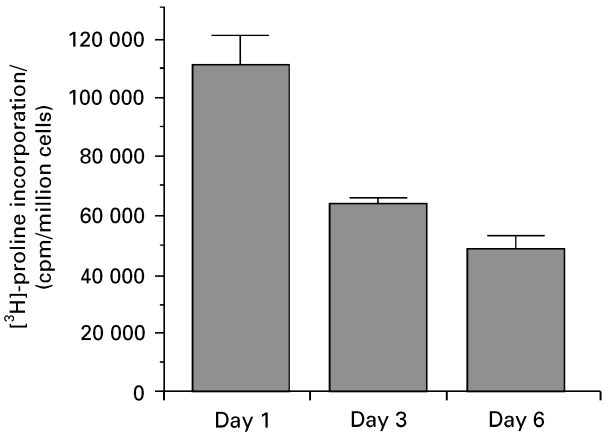

Figure 4 Tritiated proline incorporation by fibroblasts within collagen gels measured on day 1, day 3 and day 6 of culture by precipitation of pepsin-digested samples with 1% sirius red in distilled water. Each value represents the mean and standard error of three replicates.

Previously, 1% sirius red in picric acid has been demonstrated to bind specifically to molecules containing the typical collagen triple helical structure but the inclusion of picric acid is problematic due to the precipitation of non-collagenous protein species. In the present study, we have determined that binding of sirius red to purified types I, II and III collagen solutions was not altered by using either saturated aqueous picric acid or water as solvents for the dye. These findings suggest that whilst picric acid may be advantageous in a histological method, where it both fixes the tissue and provides a pale yellow counterstain, it is not necessary for collagen binding *per se*. Previous suggestions that picric acid was necessary for the specificity of the dye [12] proved unfounded with considerably reduced binding of sirius red in water to serum components in comparison with sirius red in picric acid (results not shown).

Sirius red (1%) in either picric acid or water and 10% TCA, all precipitated labelled material which was specific to macromolecular protein as indicated by the eradication of measured incorporation when the cells were incubated in the presence of cycloheximide. Significant precipitation of labelled material was detected, however, using picric acid alone, indicating that the precipitation with 1% sirius red in picric acid is not specific to the dye. This is to be expected, as picric acid may be used as a precipitating fixative for histology. Precipitation was dye specific for 1% sirius red in water as indicated by the lack of precipitation of labelled material by water alone.

It has been suggested that sirius red may bind non-specifically to proteins containing poly-basic amino acid regions, such as arginine- and lysine-containing histones, by electrostatic interactions [16]. In order to determine the selectivity of precipitation for collagen using this method, labelled gels were treated with collagenase prior to assay. The collagenase used is a commercially available highly purified preparation which has been tested for caseinase (none detected), tryptic (none detected) and clostripain (0.086 μunit/ unit collagenase) activity suggesting that non-specific activity is negligible. Both 1% sirius red in picric acid and 10% TCA demonstrated significant precipitation of labelled material after collagenase treatment, indicating a lack of specificity for collagenase-sensitive components. Precipitation was almost totally eradicated by collagenase when using 1% sirius red in water, indicating a high degree of specificity for collagenous components using this method. Concerns regarding non-specific precipitation proved to be unfounded, therefore, when using the method described. The specificity of this method would appear to be due to a combination of labelling with proline, which comprises approximately 22% of total amino acid residues (including hydroxyproline), the formation of non-precipitating fragments of pepsin-sensitive proteins, and the inherent specificity of the 1% sirius red/water preparation.

The method was used to determine the rate of collagen synthesis by dermal fibroblasts cultured within freely contracting collagen gels for up to 6 d. The results indicated a down regulation of collagen synthesis with time in culture which agrees with previous studies measuring collagen synthesis by other methods [17]. The results demonstrate that 1% sirius red in water may be used to precipitate [^{3}H]-proline incorporated into newly synthesized collagen in a highly specific manner. The method can be used to assess collagen synthesis in a simple and accurate manner in many cell-culture systems including collagen gels. The inherent specificity of the dye reagent means that this technique has the potential to become the method of choice for the assessment of collagen synthesis by cells in collagen-based biomaterials *in vitro*.

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