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Collagen Stimulating Effect of Peptide Amphiphile C_{16}–KTTKS on Human Fibroblasts

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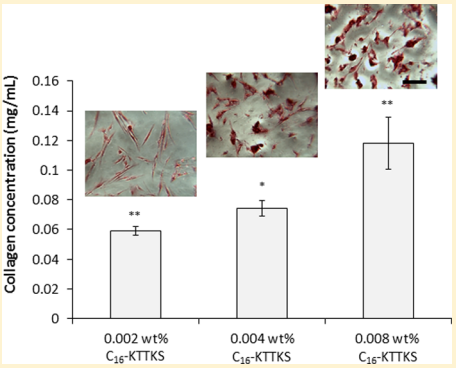
ABSTRACT: The collagen production of human dermal and corneal fibroblasts in contact with solutions of the peptide amphiphile (PA) C_{16}–KTTKS is investigated and related to its self-assembly into nanotape structures. This PA is used in antiwrinkle cosmeceutical applications (trade name Matrixyl). We prove that C_{16}–KTTKS stimulates collagen production in a concentration-dependent manner close to the critical aggregation concentration determined from pyrene fluorescence spectroscopy. This suggests that self-assembly and the stimulation of collagen production are inter-related.



KEYWORDS: *peptide amphiphile, Matrixyl, collagen, fibroblasts, skincare*

INTRODUCTION

The development of materials for tissue engineering applications in regenerative medicine is a major challenge currently attracting intense research activity at the interface of synthetic and biomaterials chemistry. Collagen is the most abundant protein in mammals and constitutes a significant proportion of connective tissue. Collagen is a key component of the extracellular matrix (ECM), and so, controlling its production and properties (e.g., type, fibril dimensions, and arrangement or alignment) is a key aspect in tissue engineering. Collagen substrates are also widely used to culture cells for stem-cell research.[1] Collagen-based materials are extensively used for skin[2] and corneal[3] tissue engineering, for instance, for wound healing; an excellent review provides examples of commercial products in development for this and related applications for other tissue types.[2] A current commercial application of collagen-stimulating peptide amphiphiles is in the formulation of skincare products, where they have been shown to provide multiple skin health and appearance benefits.[4−7]

Several synthetic peptide-based systems have been developed for cell culture applications including the commercially available material PuraMatrix, based on the self-assembling peptide RADA-16I [Ac-$(RADA)_4$-$CONH_2$], which forms a fibrillar hydrogel that has demonstrated efficacy in stimulating cell growth and for tissue scaffolding.[8−10] Significant research activity is underway on other synthetic systems designed to mimic the ECM for applications in tissue engineering, as reviewed elsewhere.[11,12] A complex combination of properties is demanded of these materials, including appropriate binding sites, signaling elements, and suitable matrix elasticity.[1,2] A common bioderived substrate is Matrigel, which is a secreted extract from mouse tumor cells.[11,13] Within the academic and commercial patent literature there are several publications on peptides developed to stimulate collagen production for skincare applications. A particular system, currently in widespread commercial use, is the peptide amphiphile (PA) C_{16}–KTTKS with the trade name (registered to Sederma SA, France) of Matrixyl. C_{16}–KTTKS is a lipidated peptide with a pentapeptide "headgroup" based on a sequence taken from a propeptide from Human type I collagen.[4,14−16] It has been reported that both the peptide KTTKS[14] and C_{16}–KTTKS are able to promote type I collagen production in in vitro cell culture studies. This is further supported by clinical studies, which suggest that C_{16}–KTTKS can reduce the appearance of facial wrinkles.[4−6]

An important consideration in topical application of peptide amphiphiles is transport of the active type across the stratum corneum, and with this in mind, lipidation of the peptide KTTKS was carried out in order to improve delivery across the epidermis.[17,18] For significant concentrations of peptide to penetrate the stratum corneum, it would be necessary to disrupt the stratum corneum through microdermabrasion or similar physical trauma. Alternatively, direct injection into the dermis could be used, as in the case of dermal fillers such as Botox,[19,20] although currently all of these methods are largely undesirable for the majority of skincare consumers. The study of transport across the stratum corneum is beyond the scope of this article. In future work, we will be examining this and also the use of peptide amphiphiles such as C_{16}–KTTKS in medical applications where these materials could find valuable

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application for instance in topical application to the damaged skin of burns patients or open wounds.

We recently investigated the self-assembly of C_{16}–KTTKS in aqueous solution[15] as well as its mixtures with surfactants.[21,22] Tape-like nanostructures were observed, with a broad distribution of widths. The highly extended tape-like structures aggregate into fibrillar bundles, which are large enough to be imaged by optical microscopy. The nanostructure of the tapes was probed by cryo-TEM and small-angle X-ray scattering (SAXS). SAXS on an aligned sample was performed to establish that the internal structure of C_{16}–KTTKS nanotapes comprises PA bilayers.[15] We have also investigated the self-assembly of the related PAs C_{14}–KTTKS and C_{18}–KTTKS with different lipid chain lengths.[23]

It has been claimed that C_{16}–KTTKS in contact with human dermal fibroblast (HDF) cells is able to stimulate type I collagen production.[4,5] This result was partially concluded from collagen I assays using an ELISA method, using 10^{-6} wt % transforming growth factor β as a positive control product for samples containing $(1-8) \times 10^{-4}$ wt % C_{16}–KTTKS.

In this work, we report on collagen production assays, culturing human corneal fibroblast (HCF), and human dermal fibroblast (HDF) cells in the presence of C_{16}–KTTKS at PA concentrations higher than those examined previously since we also intend to obtain an insight on the possible influence of PA self-assembly on the collagen production. We seek to investigate the production of collagen by human fibroblasts in the presence of $(2-8) \times 10^{-3}$ wt % C_{16}–KTTKS.

The biological synthesis of collagen can be assayed through a Sirius red assay. Sirius red in picric acid solution has been used for quantification of collagen in tissue sections for many years.[24−28] This method is based on the observation that Sirius red in saturated picric acid selectively binds to fibrillar collagens (types I to V) and the mechanism of binding is thought to involve the acidic interaction of the dye with the basic amino acid residues present in collagen (Gly-X-Y).[29] HDF cells exposed to ascorbic acid synthesize about ten times more type I than type III collagen.[30] In addition, contents of collagen types different from I and III originate from the HDF cells alone, so they will contribute as a constant factor for all the samples used in the Sirius red assay.

Following collagen production assays, we measured the critical aggregation concentration (*cac*) of C_{16}–KTTKS solutions in cell culture media using pyrene and Thioflavin T fluorescence techniques. The former is sensitive to changes in the hydrophobic environment associated with self-assembly,[31,32] whereas the latter is diagnostic of amyloid β-sheet formation.[33]

In the following, we put forward a hypothesis that bioactivity of the C_{16}–KTTKS PA is significantly influenced by its self-assembly. The formation of self-assembled aggregates would be expected to create organized nanostructures such as tapes or fibrils that present their peptide motifs at the periphery. This may substantially impact their activity. We find that there is a dose-dependent increase in collagen production by C_{16}–KTTKS around the *cac*.

EXPERIMENTAL SECTION

Materials. Peptide amphiphile C_{16}–KTTKS, Palmitoyl-Lys-Thr-Thr-Lys-Ser was purchased from CS Bio (US). Purity was 97.6% by analytical high-performance liquid chromatography (HPLC), M_w 802.47 Da (expected), 802.05 Da (measured), acetate content was 11% (by HPLC). HDF cells were purchased from Invitrogen (U.K.). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), antibiotics, fungizone, and tissue culture plates were obtained from Fisher Scientific (UK). Pyr, L-ascorbic acid, and all reagents for Sirius red staining used for the Sirius red assay were purchased from Sigma-Aldrich (U.K.).

Preparation of Human Corneal and Human Dermal Fibroblasts. Human corneal rings comprising the conjunctiva, limbus, and central cornea were obtained from the Royal Berkshire Hospital and dissected into 4 pieces. Excess conjunctiva and central cornea were mechanically removed using a scalpel. The remaining stromal tissue was minced with a blade and digested in 0.25% Trypsin/EDTA solution (Fisher Scientific, U.K.) at 37 °C for 2−3 h. The digest was then purified through a sterile cell strainer (Fisher Scientific, U.K.) and centrifuged at 1500 rpm for 5 min. The pellet was resuspended in DMEM F12 medium containing 5% fetal bovine serum (FBS) and maintained at 37 °C until confluent. Adult human dermal fibroblasts purchased from Gibco (Invitrogen, U.K.) were mainlined at 37 °C until confluent.

Preparation of PA Solutions. To determine the stimulatory effects of C_{16}–KTTKS on collagen production in human dermal fibroblasts and human corneal fibroblasts, three concentrations of the peptide amphiphile were used (0.002, 0.004, and 0.008 wt %). The sample in powder form was weighed out, treated under UV light in a Biological class II safety cabinet (Triple Red, UK) for 30 min prior to mixing with DMEM basal media. and sonicated for 10 min to dissolve the peptide.

Culture of Human Fibroblasts with Matrixyl. HCF and HDF were plated at a density of 9×10^3 cells per well into 24 well tissue culture plates (n = 3) in basal DMEM F12 (1:1) media supplemented with 5% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin with 0.25 μg/mL Amphotericin B (Fisher Scientific, UK), and 5 μg/mL Insulin (Invitrogen, U.K.) overnight to allow the adherence of cells to tissue culture plates. The following day, culture medium was removed and replaced with three concentrations of Matrixyl. Controls in basal media (DMEM F12 with no supplements) and conventional additives (1 mM ascorbic acid, 0.025 ng/mL TGFβ1, and 0.01 mg/mL Insulin) were used. Each treatment was added in duplicate and experiments were carried out for the duration of 3 days. Conventional additives were chosen based on the stimulation of HDF cells type I and III collagen synthesis by L-ascorbic acid, previously reported in the literature.[13,30,34−37]

MTT Assay for Cell Viability. The principle of the MTT assay involves the conversion of water-soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to insoluble formazan by viable cells. The quantity of viable cells can be determined by colorimetric analysis of formazan as cells metabolize MTT.

Following 3 days of incubation with Matrixyl and collagen stimulating additives, culture medium was removed and replaced with phenol-free DMEM (Fisher Scientific, U.K.). MTT solution (Sigma Aldrich, U.K.) was added to each well, and cells were incubated for 4 h at 37 °C. Negative controls of MTT solution and culture media with no cells were included. Following cell labeling with MTT, dimethyl sulfoxide was added to each well and mixed using an orbital shaker prior to incubation for a further 10 min at 37 °C. Samples were mixed, and the optical density of formazan produced by the cells was read at an absorbance of 540 nm on an Emax precision

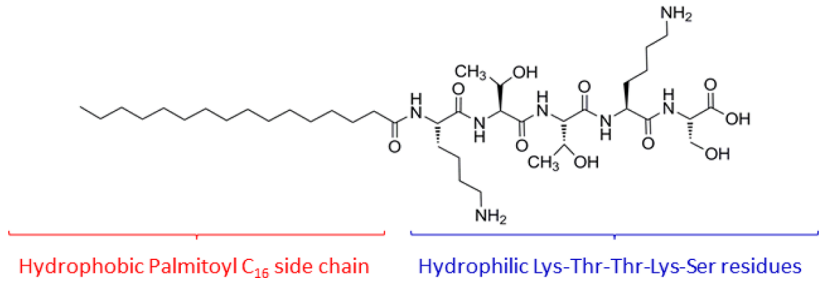

Figure 1. Molecular structure of C_{16}–KTTKS.

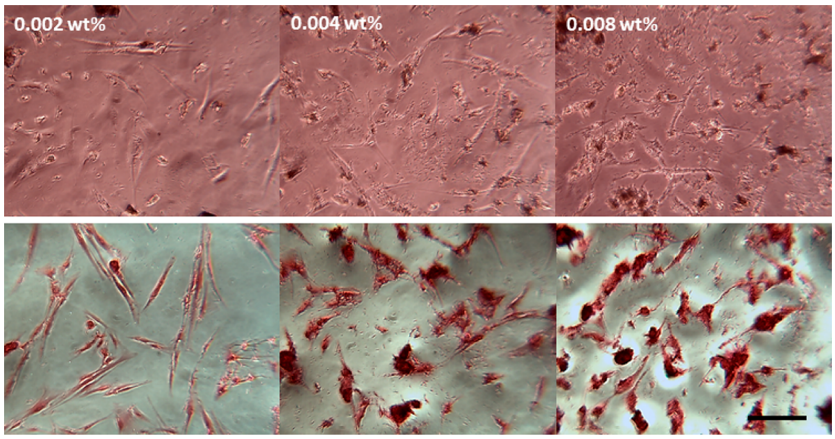

Figure 2. Representative images of Sirius red uptake of deposited collagen by human corneal fibroblasts (scale bar = 150 μm). Top panel shows light micrograph images of cells cultured with C_{16}–KTTKS; bottom panel shows Sirius red dye uptake by cells following histological staining in situ.

microplate reader (Molecular Devices, U.K.). A standard curve was constructed by plating known quantities of each cell type and the protocol for MTT cell labeling was followed. Initial cell number was plotted against absorbance readings at 540 nm in Excel where a standard curve was generated with a line of best fit. On each occasion that the MTT assay was carried out, a new standard curve was generated. The gradient from each curve was used to calculate the number of viable cells in each well. For all samples including standards, blank readings were taken, and the absorbance was adjusted accordingly.

Analysis of Collagen Production. Following 3 days in culture, media was removed and the quantity of collagen deposited by cells was measured. Cells were fixed in situ with 70% ice cold ethanol to fix deposited material to the surface of tissue culture plastic. Samples were transferred to −80 °C for a minimum of 10 min to ensure complete fixation of material. The ethanol solution was then removed, and each well was washed with distilled water. Cells were treated with Sirius red-picric acid solution and incubated overnight at 4 °C with gentle agitation. The following day, unbound dye was removed by gently rinsing the fixed cells with distilled water. This process was repeated until residual unbound dye and excess water was removed from the bottom of each well. Cells were then treated with 1 M NaOH at room temperature with gentle agitation for 10 min to dissolve the collagen dye complex and ensure thorough mixing. Then, 100 μL aliquots of each sample were transferred in duplicate to a 96 well microplate and the absorbance measured at 490 nm on an Emax precision microplate reader (Molecular Devices, U.K.).

A standard curve was constructed by drying known concentrations of rat-tail collagen type I (First Link Ltd., U.K.) on the surface of tissue culture plastic. Standard samples were treated with Sirius red and measured using the protocol described above. Absorbance values against standard collagen concentrations were plotted into Excel, and a line of best fit was generated for each standard curve. Blank readings were taken for all samples including standards, and the absorbance was adjusted accordingly.

Data Analysis. Data obtained from the MTT assay for human corneal and human dermal fibroblasts was used to calculate the effects of C_{16}–KTTKS on cell number from day 0 to the end of the experiment. Total cell number for each cell type and each treatment condition was used to normalize the amount of collagen produced per cell in each sample.

Data collected from the collagen production assay was utilized to calculate the concentration of deposited collagen by each cell type. Statistical analysis was carried out in GraphPad Prism 5 using One-way ANOVA (n = 3), and results are presented with a standard deviation of the mean where $P \leq 0.05$ was considered significant.

Light Microscopy. The overall organization of cultured samples was studied after three days of incubation using phase contrast microscopy. Cultured samples, contained in wells, were viewed under phase illumination with an inverted microscope Motic AE21. Images were recorded using a Moticom 2000 camera.

Pyrene (Pyr) and Thioflavin (ThT) Fluorescence Spectroscopy. Spectra were recorded on a Varian Cary Eclipse Fluorescence Spectrometer with samples in quartz cells with an internal width of 4 mm. Basal media (DMEM F12 with no supplements) was used, instead of water, to dissolve Pyr and ThT.

For Pyr assays, a dried film was obtained by evaporating ethanol from a 0.1 wt % solution. The Pyr dried film was then

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resuspended to give a 3.8×10^{-5} wt % Pyr solution in basal media. C_{16}–KTTKS solutions containing ($2–9 \times 10^{-3}$) wt % PA were prepared using 3.8×10^{-5} wt % Pyr in media as a solvent. Pyr fluorescence emission spectra were measured from 360 to 600 nm, using λ_{ex} = 339 nm. The fluorescence emission of media was subtracted from the fluorescence emission of the C_{16}–KTTKS/Pyr mixtures.

For the ThT assay, C_{16}–KTTKS solutions containing ($1–9 \times 10^{-3}$) wt % peptide were dissolved in 2.3×10^{-3} wt % ThT in basal media. ThT fluorescence emission spectra were measured from 460 to 600 nm, using λ_{ex} = 440 nm. The fluorescence emission of 2.3×10^{-3} wt % ThT in basal media was subtracted from the fluorescence emission of the C_{16}–KTTKS/ThT mixtures.

RESULTS AND DISCUSSION

The bioactivity of C_{16}–KTTKS relates to the pentapeptide headgroup (Figure 1) based on the amino acid sequence from the pro-peptide from human collagen type I.[23,38] We have previously suggested that the mechanism of action involving the stimulation of collagen relates to the presentation of peptide epitopes on the surface of self-assembled nanotapes.[15] The self-assembly of these nanotapes may increase the bioactivity of C_{16}–KTTKS by enabling cells to recognize the procollagen residue and subsequently lay down collagen. Our studies seek to investigate whether the bioactivity of Matrixyl increases the stimulation of collagen production in both human dermal and human corneal fibroblasts.

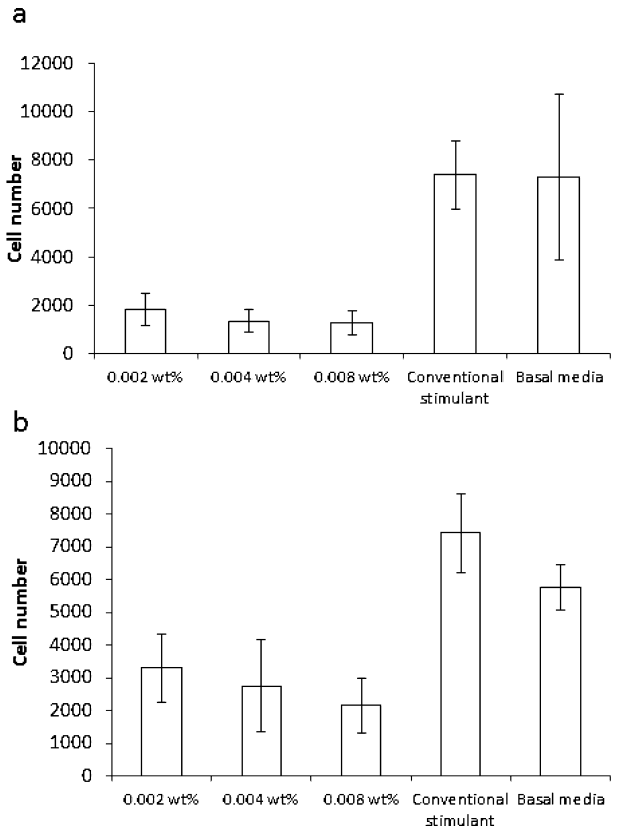

Figure 3. Cell number for (a) human corneal fibroblasts and (b) human dermal fibroblasts following treatment with C_{16}–KTTKS, conventional stimulants, and basal media at 37 °C for 3 days.

The production of collagen by human corneal and human dermal fibroblasts was investigated using the Sirius red assay. Sirius red has previously been used in the histological staining of collagen,[24–29] and we show the uptake of Sirius red dye in corneal fibroblasts following the treatment with C_{16}–KTTKS for 3 days (Figure 2). In the bottom panel, we can see an increase in the intensity of the staining based on the uptake of the dye in deposited collagen. The top panel shows cells in culture prior to staining, and we observed typical fibroblastic morphology in cells treated with 0.002, 0.004, and 0.008 wt % C_{16}–KTTKS. This suggests that cell phenotype remained unchanged throughout the course of experiments and that the PA did not have any adverse effects on cell morphology.

To accurately measure the effects of C_{16}–KTTKS on collagen production, cells were cultured minus the addition of growth stimulating additives. Figure 3 shows the effects of C_{16}–KTTKS on cell number. Compared with the proliferative capacity of conventional collagen stimulants and basal media, we observed a decrease in the number of cells following initial cell seeding. However, there was no significant difference in final cell number between the three concentrations of C_{16}–KTTKS.

In addition to histological staining, Sirius red has been used for the quantitative analysis of deposited collagen in tissues and cells;[26–28] therefore, this method was used to investigate the concentration of collagen deposited by human dermal and human corneal fibroblasts in response to stimulation by C_{16}–KTTKS (Figure 4). The results indicate a significant increase in the total amount of collagen deposited by human corneal fibroblasts treated with C_{16}–KTTKS (Figure 4a) and more specifically, cells treated with 0.008 wt % C_{16}–KTTKS produced 1.7 times more collagen (0.118 mg/mL) than corneal fibroblasts in basal media (0.07 mg/mL). In addition, there was a marked dose-dependent increase in the concentration of collagen produced in cells with the three concentrations of C_{16}–KTTKS (Figure 4a). The results also show a considerable difference in the concentration of collagen produced per individual cell across all C_{16}–KTTKS treatment groups (Figure 4b), which again appeared to be dose responsive.

The effects of C_{16}–KTTKS on human dermal fibroblasts are shown in Figure 4c,d. A marked dose response to the PA by dermal fibroblasts (Figure 4c) was observed, and dermal fibroblasts cultured with 0.008 wt % C_{16}–KTTKS were stimulated to produce a significantly higher concentration of total collagen than those treated with the conventional stimulant or basal media. Figure 4d shows a two-fold increase in the amount of collagen produced per cell from 0.004 to 0.008 wt % C_{16}–KTTKS at 0.03 and 0.06 mg/mL, respectively, and there is an obvious surge in the concentration of collagen produced per cell compared with the conventional stimulant and basal media.

Our findings, although preliminary, are particularly interesting as they suggest that, while C_{16}–KTTKS appears to have some inhibitory effects on cell proliferation, the stimulatory effects on the production of collagen were so significant that, despite a relative decrease in cell number, the concentration of collagen produced by both human dermal and human corneal fibroblasts were significantly higher than that produced using conventional stimulants (Figure 4b,d). This is in contrast to alternative conventional stimulants that have previously been shown to stimulate cell proliferation.[39–41] Increases in cell density are likely to ultimately impact the final concentration of

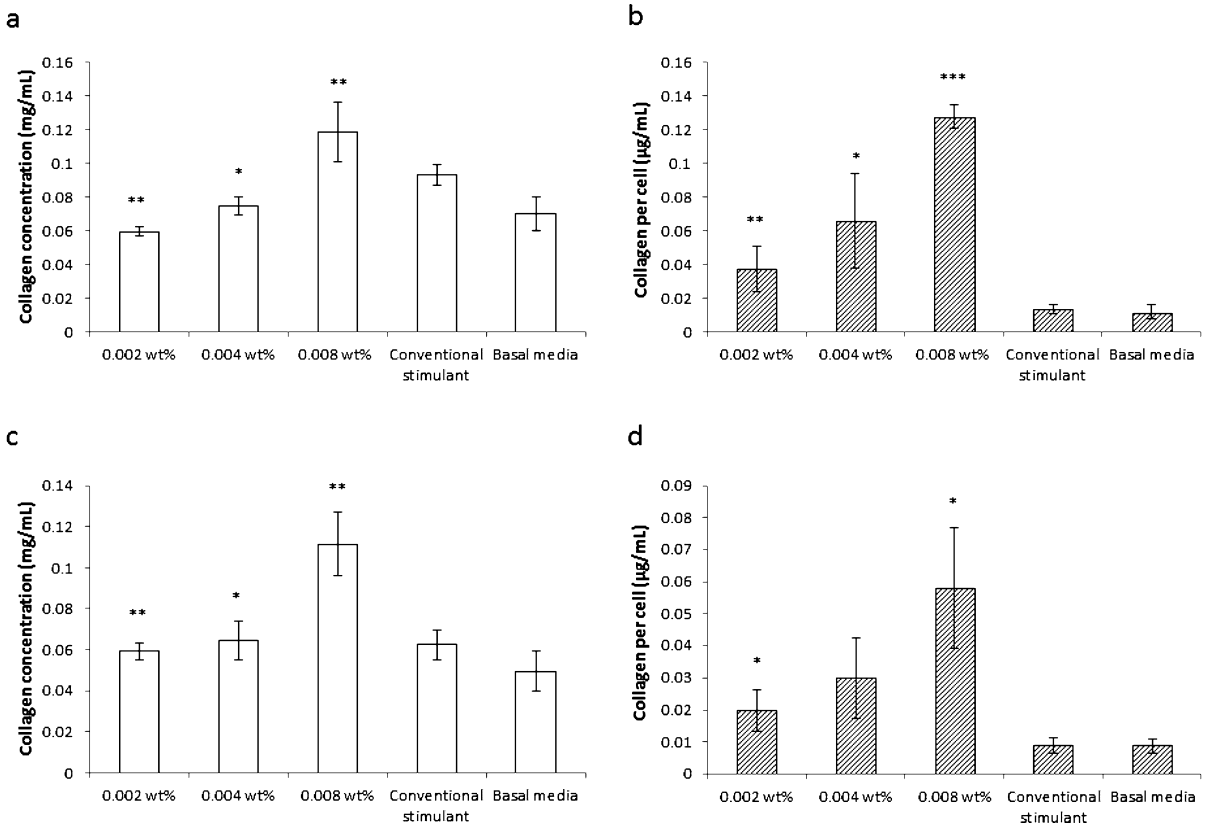

Figure 4. Graphs showing the stimulation of collagen in human corneal (a,b) and human dermal (c,d) fibroblasts by C_{16}–KTTKS. Left, total amount of collagen deposited by cells, and right, total amount of collagen produced per cell (n = 3; $^*P \leq 0.005$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$).

collagen produced, therefore masking the collagen stimulating effects of the additives.

Our objective was to relate the collagen stimulation and cell proliferation to the self-assembly of C_{16}–KTTKS. With this goal, the *cac* of C_{16}–KTTKS was determined by pyrene and thioflavin T fluorescence spectroscopy. Pyr fluorescence experiments were performed on C_{16}–KTTKS solutions in basal media at 37 °C. The emission spectrum of Pyr (λ_{ex} = 339 nm) is characterized by the strong fluorescence of the 0–0 band ($\lambda \approx 373$ nm), denoted as I_1 (inset in Figure 5a).[31,32,42] Changes in I_1 reflect the variations of the lifetime of the excited state of Pyr, which is significantly altered for different probe surroundings.[43] For amphiphilic solutions, a substantial increase in I_1 upon adding amphiphile to the solution is due to the transfer of the amphiphile to a less polar domain (i.e., micellar core).[43] This allows the *cac* to be determined from the dependence of I_1 on the amphiphile concentration.

The inset in Figure 5 shows fluorescence emission intensities from pyrene and thioflavin T spectra recorded for samples with varying concentration of C_{16}–KTTKS. Discontinuities in I_1 in the pyrene spectra (representative spectra shown in the inset) revealed a *cac* = 0.0056 wt %. The thioflavin T data reveal a *cac* = 0.0054 wt %, in very good agreement with that obtained from pyrene fluorescence. Since these two techniques probe distinct aspects of self-assembly, we can conclude that aggregation occurs with concurrent formation of hydrophobic fibril cores (probed by pyrene) and β-sheet structure of the pentapeptide headgroups (probed by ThT). Following a procedure similar to that used to obtain the results in Figure 5, the *cac* was found to be 0.03 wt % C_{16}–KTTKS for aqueous solutions of the peptide measured at room temperature (results not shown). DMEM has a high inorganic salt content, which may screen the charge on C_{16}–KTTKS and shift the *cac* to lower concentrations.

CONCLUSIONS

We investigated the production of collagen by HCF and HDF treated with C_{16}–KTTKS for 3 days. Only one treatment group with the highest concentration studied (0.008 wt % PA) was shown to stimulate collagen production to a level higher than controls.

Pyrene and thioflavin T fluorescence spectroscopy provided consistent values for the critical aggregation concentration of C_{16}–KTTKS in DMEM media at 37 °C, *cac* = (0.0055 ± 0.0002) wt %. It has previously been suggested that the synthesis of collagen and cell proliferation in fibroblasts may be linked.[30,44] Our results are not only consistent with the hypothesis that C_{16}–KTTKS collagen synthesis and growth are associated but also show that the collagen production of C_{16}–KTTKS increases in a dose-dependent manner close to the *cac* (Figure 4). It has been reported that the ECM comprises small parallel arrays of polydisperse fibrils in cell cultures of human corneal fibroblasts in contact with L-ascorbic acid.[40] It is possible that above the *cac*, the PA aggregates act as a suitable scaffold to stabilize the collagen generated in the ECM by the cells into parallel arrays. However, establishing a clear correlation between aggregation and collagen stimulation would require measurement of additional data points. Nevertheless, these findings provide an important insight into the activity of this PA and may facilitate the development of novel biomaterials or native tissue scaffolds for regenerative medicine applications, such as those involving wound healing.

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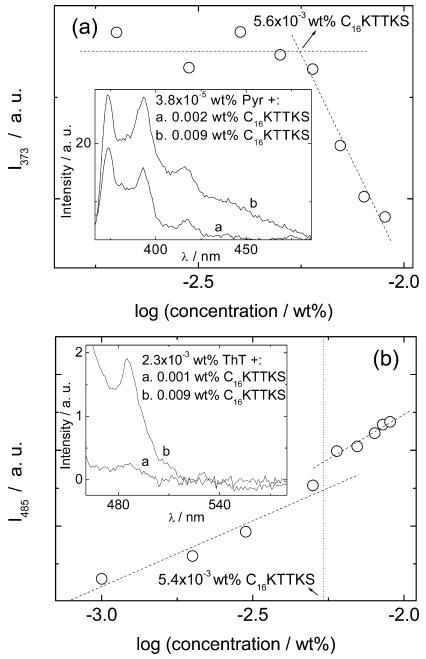

Figure 5. Fluorescence emission spectral intensities used to determined the critical aggregation concentration. (a) Intensity I_1 (373 nm) as a function of the C_{16}–KTTKS concentration measured for 3.8×10^{-5} wt % Pyr dissolved in basal media at 37 °C. The critical concentration aggregation, *cac* ≈ 0.0056 wt % C_{16}–KTTKS, is indicated. The inset shows representative spectra. (b) Intensity *I*(485 nm) as a function of the C_{16}–KTTKS concentration measured for 2.3×10^{-3} wt % ThT at 37 °C. The critical concentration aggregation, *cac* ≈ 0.0054 wt % C_{16}–KTTKS, is indicated. The inset shows representative spectra.

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Notes

The authors declare no competing financial interest.

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