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Collagen Crosslinking with Au Nanoparticles

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Abstract

Tiopronin (N-(2-Mercaptopropionyl)glycine) protected gold nanoparticles (TPAu) were crosslinked to collagen *via* EDC (1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide) coupling. On average, each TPAu forms 8 amide bonds with collagen lysine moieties. The resulting gels were studied with Environmental-SEM, TEM, micro-DSC, and TNBS assay. The porous structure of collagen was significantly altered by crosslinking, resulting in the reduction of the pore size from ca. 140 μm to less than 1μm depending upon the concentration of nanoparticles. The collagenase biodegradation assay showed improved stability of crosslinked material. The cell viability assay, CellTiter 96®, indicates that the gold nanoparticles are not toxic at the concentrations used in gel synthesis. This new material has potential for the delivery of small molecule drugs, as well as Au nanoparticles for photothermal therapies, imaging, and cell targeting.

Keywords

collagen gel; Au nanoparticles; crosslinking; microstructure

1. Introduction

Collagen is the most abundant protein and a major component of connective tissue.[1] It is also one of the most common biomaterials with broad applications ranging from drug delivery, to tissue-engineering, scaffolds, and materials for wound dressing.[2,3] Numerous medicinal applications of collagen result from its excellent biocompatibility, biodegradability, and the ease of extraction, purification and processing.[4]

However, the biodegradation rate and the mechanical stability of native collagen are not sufficient for many *in vivo* and *in vitro* applications. The crosslinking of collagen is one way to improve the mechanical stability and to slow down the biodegradation rate.[1,5,6] For these reasons several crosslinking procedures including reactions with glutaraldehyde, isocyanates, epoxides, and bis imidates, as well as thermal treatment, UV or gamma-ray irradiation, and photo-oxidation have been developed.[6,7] Many of the chemical crosslinking methods incorporate a “bridge” molecule as a part of the crosslink. Another way to chemically crosslink collagen is to use so called “zero-length” linkers; coupling agents capable of forming peptide bonds between collagen molecules. EDC (1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide) is one such a coupling agent.[5,8,9]

Both types of crosslinking schemes produce the “two-point” connections between the collagen molecules, thus irrespective of method the crosslinking products bear some structural similarities.

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It appears that the use of the multiple-way crosslinkers should result in development of collagen materials exhibiting novel properties. Interestingly, natural crosslinking occurs as collagen ages.[10] The pyridinoline crosslink is thought to be responsible for structural and mechanical differences between young and aged collagen.[11,12] This crosslink provides a 3-way connection - three side groups of collagen connected together.

Ramanujan et al. have shown that restricting the collagen pore size by fibrilogenesis in high concentration of collagen can lead to collagen gels better suited for small molecules delivery.[13] De Paoli Lacerda et al. reported recently that there is a correlation between the collagen gel morphology and release rate of encapsulated small molecules.[14] Thus there is an interest in synthesizing new collagen materials with decreased porosity with potential applications as effective carrier for small molecules delivery.

Recently Duan et al have shown that the use of a dendrimer with 4, 8, and 16 amino groups was crosslinked to collagen using EDC to produce collagen gels with thermal stability that is better than for the glutaraldehyde crosslinked gels.[15] Moreover the multiple-way crosslinks should allow incorporation of multiple functionalities within the matrix in one step.[15–17]

Here we propose a novel approach to collagen crosslinking based on tiopronin modified gold nanoparticles. Multiple carboxyl groups are present on the surface of Au nanoparticles thus nanoparticles are capable of forming the multiple crosslinks with the collagen. The formation of the peptide bonds with amino groups of collagen is achieved *via* EDC coupling.

Au nanoparticles are known to be biocompatible and can be easily functionalized with biomolecules i.e. growth factors, DNA, peptides etc.[18] Additionally they are frequently used as drug delivery system or to target the specific cells.[19,20] Moreover the size and shape of Au nanoparticles can be widely adjusted. Au nanoparticles were extensively studied as new contrast agents for imaging and novel photothermal therapies.[19–23] The latter one is recently gaining a lot of interest due to rapid technological advances in synthesis of hollow Au particles of different shapes and sizes, and the possibility of organization of small Au nanoparticles into well defined clusters.[21] These advances lead to maximizing the light absorption in near infrared NIR, thus increasing the power of photothermal therapies. Thus the collagen materials that incorporate Au nanoparticles can also be considered as a vehicle for the particle delivery.

Recently, Hsu at al. showed that porcine type II collagen and gold colloid nanocomposite have potential use in cartilage tissue engineering due to enhancement in material modulus, chondrocyte growth, and antioxidant effect.[24] The application of Au nanoparticles as a crosslinking agent in collagen gels also allows for easy incorporation of biomolecules (growth factors, cell adhesion molecules and peptides) by their immobilization at the Au surface without additional altering of collagen structure.

The results described below show that EDC assisted crosslinking of collagen with TPAu results in a formation of about 8 bonds between each nanoparticle and collagen molecules. Furthermore, the ratio between the concentrations of collagen and nanoparticles strongly influences the gel pore sizes. The resulting gels show improved stability against biodegradation and are biocompatible.

2. Materials and Methods

2.1 Preparation of Au nanoparticles

Tiopronin protected gold nanoclusters (TPAu), diameter = 2.4 (± 0.7) nm, were synthesized using known methods.[25,26] Briefly, 0.31g tetrachloroauric acid (Sigma) and 0.39 g N-(2-mercaptopropionyl)glycine (Aldrich) were slowly dissolved in 35 ml of 6:1 methanol/acetic

acid solution, to this 0.6g $NaBH_4$ (Sigma) was added in 15 ml of water. The resulting hot, blue-black suspension was cooled down to room temperature and stirred for 30 minutes. The crude black TPAu was purified by dialysis, with fresh water recharged every 14 hours over the course of 70 hours. The nanoparticles were characterized by spectrophotometry, thermogravimetric analysis and transmission electron microscopy.

2.2 Collagen crosslinking

The collagen was extracted from rat tail tendons (Pel-Freeze Biologicals) according to a procedure by Ho et al., lyophilized and stored at 4°C until used.[27] The yield of this procedure was 29–31%. The collagen was then dissolved in 0.01 M HCl and the purity of the product was examined by standard SDS-PAGE.

The tiopronin modified gold nanoclusters (TPAu) were coupled to the collagen *via* EDC (N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride, Aldrich) coupling. Briefly, 600μL of 1% (w/v) stock solution of collagen in DI water was added to varied amounts (40–360 μL) of 30 mg/mL TPAu in DI water (Millipore). The pH of a resulting solution was about 6 and was constant in the course of the coupling reaction. Subsequently, 30 mg of EDC (1.5×10^{-4} M, what reflects 10 times excess with respect to carboxyl groups to be activated, when the highest concentration of nanoparticles is used for collagen crosslinking) was added to the solution and the volume was adjusted to 1.5 mL with DI water. The sample was left overnight to crosslink. The crosslinked matrix appeared almost solid, blue and opaque.

2.3 Microstructure observation

The low concentrations of the collagen solutions used in the preparation of the collagen gels led to the weak gels, thus the microstructure characterization of the samples were performed on the freeze-dried gels. The freeze-dried gel samples were analyzed with Environmental Scanning Electron Microscope (SEM) (FEI Quanta 200). The samples were cross-sectioned and coated with gold (Cressington 309 metal evaporator)

Transmition Electron Microscopy (TEM) of Au nanoparticles samples were prepared by placing a drop of water solution with Au-Tp on the Formvar-coated copper grids (1–3mg/ml). Phase contrast images were recorded at 80 KeV with JEOL electron microscope. Because spreading of freeze-dried collagen gels crosslinked with Au nanoparticles on the TEM grid proved difficult, gels were dried with progressively increasing concentration of ethanol solutions, cured in the Araldite (Ted Pella) resin and sectioned (LKB Ultratome Nova) to 60–80 nm thickness before image.

2.4 Determination of cross-linking degree

The degree of cross-linking was measured as a loss of free ε-amino groups of lysine upon addition of Au nanoparticles. The amount of free amino groups was determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS, Pierce) as described by Cheung et al. and Sheu et al..[28,29] Briefly, 100 μl of collagen samples treated with different amounts of Au nanopatrticles was added to 400 μl of 0.1 M sodium bicarbonate solution and 250 μl of 0.1% TNBS (in 0.1 M sodium bicarbonate) and placed in water bath (40°C) for 2 hours, 300 μl of 12M HCl was added to the reaction mixture and the temperature was raised to 60°C. The solubilization of the collagen was achieved after 60 minutes. The samples were transferred to spectrophotometer (Shimadzu UV-2401 PC) and the absorbance was measured at 425 nm (the reference sample contained appropriate amount of Au nanoparticles to avoid scattering losses). The degree of cross-linking was determined as a % loss of free ε-amino groups and is equal to: $1-(Abs_{Au}/Abs_{EDC})$, where Abs_{Au} represents the intensity of the Au cross-linked collagen and Abs_{EDC} represents the intensity of the collagen treated with EDC only.

2.5 Differential Scanning Calorimetry

The thermal properties of the collagen matrix were determined with Differential Scanning Calorimetry (DSC, MicroCal). Samples were studied 24 hours after preparation and were not lyophilized before injection. The sample size was about 2.8 mg and the scanning rate was 1° C per minute (we have also performed the measurement at faster scan rates, and the results were reproducible). The scanning temperature was 12 to 95°C and the endothermic peaks were recorded. We did not observe exothermic peaks. DI water was used as a reference. All recorded peaks represent the irreversible helix-to-coil transitions and are not present in the second scan.

2.6 In vitro collagenase degradation

A collagenase assay was performed to test biological stability of the samples. Freeze-dried collagen samples were cut into 5 mg pieces and incubated in 0.1 M tris-HCl (pH 7.4) for 1h at 37°C. 200 U of bacterial collagenase (Sigma) in 1 ml of 0.1 M tris-HCl (pH 7.4) and 0.05 M $CaCl_2$ was added to the collagen sample and incubated for 24h at 37°C. After 24h the reaction was terminated by addition of 0.2 ml 0.25 EDTA followed by cooling the mixture in an ice bath. The mixture was centrifuged for 15 min at 5000 G (4°C) and supernatant collected, autoclaved for 20 min at 125°C, and analyzed for hydroxyproline content according to the method published by Cheung et al. Briefly, the 75 μl of supernatant was added to 75 μl of freshly prepared 0.5% Chloramine T solution. After 15 minutes the 150 μl of Ehrlich reagent was added and placed in the water bath at 75°C for 15 minutes. The photometric analysis was performed by the plate reader (Variscan) at 550 nm. The liner response of the assay was determined with hydroxyproline solutions. The percentage of the degraded matrix is reported with respect to non-treated collagen.

2.7 Cytotoxicity evaluation

The cytotoxicity of the tiopronin protected Au nanoparticles was evaluated with CellTiter 96® Aqueous (Promega), colorimetric viability assay in NIH-3T3 Swiss Mice Fibroblasts cells culture, where different concentrations of sterilized by filtration nanoparticles were added to the media. The CellTiter 96® assay was performed every two hours (4–18 h). As a control DI water was added to the media in the amount reflecting the volume of nanoparticles solution.

3. Results and Discussion

Crosslinking of collagen has been extensively studied. Most of the crosslinking processes involve two-point link between the collagen molecules. Here we employ TPAu to serve as a linking agent because of its ability to form more then 2 bonds with the collagen molecule(s).

TPAu particles were synthesized according to the well known procedure shown schematically in Figure 1 (reaction 1).[25,26] In order to determine the size distribution of the Au particles (Figure 2) we have analyzed the TEM images by measuring the diameter of 200 nanoparticles. The average size of particle was found to be 2.4 ± 0.7 nm. Based on thermogravimetric analysis (TGA) and TEM measurements and assuming the core shape of the nanoparticle to be a non-ideal truncoctahedron we estimate that about 115 (TGA, 31.38 % organic) tiopronin groups are present on the nanoparticle surface.[26]

The crosslinking of collagen with TPAu was performed via EDC carbodiimide coupling reaction as shown in Figure 1, reaction 2. In order to promote the reaction between TPAu carboxylic groups and the collagen amino moieties while at the same time limiting the EDC promoted “self-crosslinking” of collagen we used a low concentration of collagen and large excess of EDC without NHS. The undesired side reaction occurring under these conditions results in crosslinking of two collagen molecules without involvement of TPAu is at the level of 2.3 ± 0.4 % as described later in the text.

The collagen gels prepared with varied amounts of Au nanoparticles (summarized in Table 1) had final concentration of collagen equal to 0.4% (w/v). All gels were opaque, blue, almost solid, and appear fragile. The addition of nanoparticles without EDC did not result in the formation of a gel after 24h.

The concentration of collagen used in our experiments was chosen because of the intended application of a gel as a drug delivery medium. However by increasing the concentration of collagen during gel formation, the resulting gels may exhibit higher mechanical stability and thus could be potentially used in tissue engineering applications. Studies in this direction are underway in our laboratory.

Following the crosslinking the gels were lyophilized and a cross-section of the samples were imaged with SEM. As shown in Figure 3, the porous structure of the gel depends significantly on the degree of crosslinking. Observed pore diameter for uncrosslinked collagen was 140 ± 35 μm (Figure 3A, B), and agrees very well with previously reported pore sizes for similar experimental conditions.[29,30] The addition of TPAu particles results in a significant decrease of the pore size (Figure 3 D–H). The gels containing two highest studied concentrations of Au nanoparticles exhibit pores with diameter below 1μm. The Au crosslinked gels are very fibrous and lack sheet-like structure often observed in lyophilized gels.

Transmission Electron Microscopy was used to image the organization of the Au nanoparticles in the gel. In Figure 4 we compare the gel AU1 (lowest content of nanoparticles) with AU5 (highest content of nanoparticles). The AU1 gel sample shows lack of continuity between the nanoparticles and their organization in the island-like structure. In the AU5 gel sample nanoparticles are interconnected and form snake like structure.

The extent of crosslinking can be evaluated on the basis of loss of the available free amino groups of collagen during the crosslinking process. This loss does not explicitly show the formation of a crosslink - Au nanoparticles can attach to just one lysine group without formation of a crosslink. However it was shown previously that the loss of free amino groups correlates very well with the extent of gel crosslinking.[31]

The results of the TNBS assay (2,4,6-trinitrobenzenesulfonic acid) shown in figure 5 indicate that the addition of EDC alone (at the same concentration as in the presence of Au nanoparticles) causes only about 2.3% loss of amino groups. We assume that these amino groups are engaged in intra- or inter-molecular crosslinking reaction of collagen. As mentioned earlier, this small amount of self-crosslinking with EDC was achieved by using a low concentration of collagen for the gel preparation solution. The collagen/Au nanoparticles molar ratio of 1:3 results in about 25% of free amino groups utilized while subsequent additions of Au nanoparticles AU4 and AU5 (with 1:6 and 1:9 mole ratios respectively) show over 98% of all free amino group utilized in formation of bonds.

We note that collagen crosslinking with nanoparticles containing surface amino groups could potentially result in the increased crosslinking density. Such strategy would enable the reaction between abundant collagen carboxyl groups and the nanoparticle amino groups resulting in a higher crosslinking density.[15]

DSC is a convenient method to measure the denaturation (shrinkage) temperature of collagen. Since the denaturation temperature reflects the triple helix-to-coil transition, it is affected by the extent, and probably the type of crosslinks present within the gel. Figure 6 shows the denaturation temperature for each sample. The denaturation of uncrosslinked collagen samples occurred at 38°C and was elevated to 42°C upon addition of EDC. Thermogram of both samples shows one peak. After addition of Au nanoparticles (AU1-3) the thermogram shows two well developed peaks; first around 42°C (T_1) and the second around 55°C (T_2). After subsequent

addition of Au nanoparticles (AU4 and AU5) the T_1 peak disappears while T_2 does not change significantly. Duan and Sheardown have shown that if the dendrimers with the amine terminal arms are used as crosslinking agents, thermograms had multiple peaks.[15] The authors argue that this behavior could be a result of heterogeneity of the sample due to multifunctionality of the dendrimers. In our experiment two peaks are present only in the samples crosslinked with EDC and small amount of nanoparticles. Thus it appears that the T_1 peak originates from the helix-to-coil transition of the self-crosslinked collagen while the T_2 peak represents the helix-to-coil transition of the collagen crosslinked with Au nanoparticles. It appears that the absence of T_1 peak from the thermogram is a result of a competition between the available carboxyl groups during the crosslinking. The samples that contain large amount of nanoparticles (AU4-5) have large number of activated carboxyl groups on the surface of a particle, thus they react preferentially with collagen amino groups. When the nanoparticles are not as abundant, some collagen carboxyl groups (up to 2.3%) are activated and form, in part, self-crosslinked gel.

Because the temperature of the T_2 in the samples AU1-3 is very similar to the temperature of the only peak in the samples AU4-5, it appears that it represents helix-to-coil transitions of structurally similar materials. Thus, considering Duan and Sheardown data on dendrimers one can assume that, on average, the number of bonds between the nanoparticle and collagen should be the similar in all nanoparticle crosslinked samples.[15] Moreover the denaturation temperature (T_2) does not change significantly upon the increase of nanoparticle concentration. Thus it appears that even after addition of large amounts of nanoparticles (AU4-5), the particles form crosslinks rather than just attach to collagen.

We note that the AU2 appears to have the highest denaturation temperature (about 3°C). Moreover the collagenase degradation time is smallest for AU2 albeit it is not statistically different ($p<0.01$) from other samples. The observed behavior could nevertheless indicate that AU2 forms the most stable gel. Since in AU2 only about 14% of collagen amino groups are used for crosslinking, this behavior seems analogous to that observed for some nanocomposites. Further studies in this direction are underway in our laboratory.

The analysis of data presented in Figure 5, samples AU1-3 together with the thermograms was used to estimate the number of crosslinks formed by each Au nanoparticle. It was reported earlier that the number of free amino groups in collagen type I is ca. 30 per 1000 residues.[32] The average mass of collagen type I molecule is 300 KDa.[10] The average number of crosslinks present on each nanoparticle is 7.9 ± 0.4 and does not depend on the collagen/nanoparticle ratio for samples AU1-3 (AU1=7.16, AU2=7.97, AU3=8.16). While the above estimation carries large error associated with the size dispersion of nanoparticles (29%), it appears nevertheless that TPAu certainly form multiple linkers. This multiplicity of crosslinking could explain unusually small porosity of the gels.

The samples AU4 and AU5 show almost complete lack of free amino group (Figure 5) after crosslinking we assume that all the amino moieties present in the collagen underwent crosslinking reaction. At the same time the DSC thermograms exhibit only one peak for each sample at about the same temperature (Figure 6). In all characterization methods described above, the sample AU4 and AU5 are very similar, but contain different amount of nanoparticles. Considering the argument of Duan and Sheardown that different number of crosslinks formed from common center (in author's case, dendrimer) would cause different denaturation temperatures in DSC, we had to conclude that both of the samples contain, on average, the same amount of crosslinks between one nanoparticle and collagen, and different amount of unreacted nanoparticles.[15]

The Collagenase Assay was used to study the biological stability of the samples in vitro, by exposing collagen gels to the collagenase solution for 24 hours. The results were normalized with respect to un-crosslinked collagen and are shown in Figure 7. Collagen treated with EDC only shows decrease in degradation to about 35% of collagen control sample. With the exception of AU1 sample, after addition of nanoparticles the degradation decreases to the level of 20–30% and this decrease in biodegradation is significant with respect to EDC only crosslinked samples ($p<0.01$). However it appears that the standard deviation of the measurements is large. In general, the samples AU2-5 show similar stability to enzymatic degradation.

The sterilized collagen gels were used as a coating for glass slides and NIH-3T3 Swiss Mice Fibroblasts cells culture to determine biocompatibility. The growth rates and cell viability were not significantly different from control and have been reported elsewhere.[33]

Because collagen gels are biodegradable, the Au nanoparticles will eventually be released to the organism. The cell uptake of Au nanoparticles strongly depends on their size and surface group. We have tested cytotoxicity of the nanoparticles with NIH-3T3 Swiss Mice Fibroblasts cells culture, by adding the nanoparticles to the media (after sterilization). After 12 hours, CellTiter 96® viability assay (Figure 8) shows that the particles we have used for the collagen modification (2.4 nm) have no adverse effect on cell viability up to 5 mmol/L. Addition of 10 mmol/L Au nanoparticles to the media results in about 20 % (with respect to control) of cell to stop being viable. We observe the steady decrease of viable cells with subsequent increase of concentration of nanoparticles up to concentrations tested (375 mmol/L). To put this in perspective, the concentrations of Au nanoparticles used for the formation of collagen gels in this work were equal to 10 μmol/L for AU1 and 89.5 μmol/L for AU5. Thus it is unlikely that this concentration range will cause toxicity. The level of toxicity of Au nanoparticles used in these studies was consistent with the literature data.

Results reported in Figure 8 were collected after 12h. The 96 well plate used for CellTiter96® assay does not allow for longer assay time because of the small volume of the media in each well. In this assay, after the initial cell seeding, the cell proliferation increases the number of cells within the first 8 hours followed by the stabilization of the number of viable cells (8–24h). After 24h the decline in the number of viable cells was observed in both control and the samples because of the lack of adherent area as well as acidification of the media. Chitchrani et. al. showed that if Au nanoparticles accumulate inside the cells, the uptake time is up to 7 hours, regardless of a particle size.[23] These authors also showed that for small particles (14, 50 nm), the number of nanoparticles inside the cell does not increase for particle concentrations above 6 and 12 μM respectively.[23] Interestingly, these authors reported that the cell viability (98%) was not affected by nanoparticle uptake.[23] In view of the above findings we believe that any acute toxic effects would be noticeable within 12h of exposure. It appears that the toxicity of a long term exposure in cell culture could only be a result of a slow particle accumulation inside the cells. However as shown by Chitchrani et.al., the accumulation does not change cell viability.

4. Conclusions

We have used tiopronin protected gold nanoparticles to synthesize highly crosslinked collagen gels *via* EDC coupling. TEM studies showed that the organization of the nanoparticles within the gel vary with concentration of particles. SEM data demonstrates that porosity of the gel is strongly dependent on the concentration of the nanoparticles and can be varied between ca. 140 μm to < 1μm. Micro-DSC and TNBS assay show that regardless of the concentration the nanoparticles the estimated number of bonds between one nanoparticle and amino moiety of collagen is about 8. At the same time, the above tests demonstrate progressive increase of

crosslinking degree with the increase of nanoparticles concentration. Collagenase assay indicated that the biodegradation time is similar in all samples treated with nanoparticles. Cytotoxicity level of tiopronin protected gold nanoparticles was evaluated with CellTiter 96® and showed no adverse effects for cell viability below 5 mM which agrees well with published data. We believe that the structure of the gel can be easily altered by varying not only the nanoparticle concentration, but also the size of nanoparticles and the number of active groups expressed on its surface. Because the Au nanoparticles can include other functionalities attached to its surface (biomolecules, targeting agents etc.) the collagen side groups do not have to be involved in this attachment, thus the properties of the collagen gel will not change. The characterization of Au nanoparticle crosslinked collagen gels shows that they have potential as a medium for delivery of small molecule drugs, and also for delivery of nanoparticles. The later can be potentially used in photothermal therapies, imaging and cell targeting.

Acknowledgments

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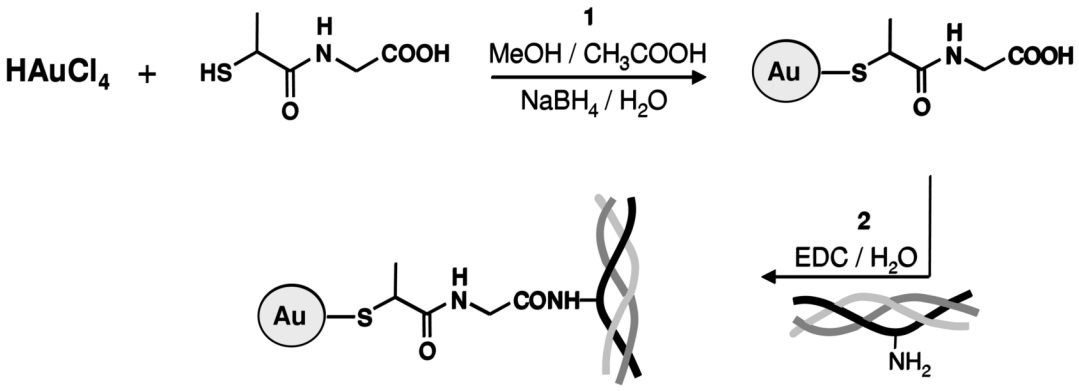

Figure 1.
Scheme of the synthesis of Au nanoparticles and crosslinking collagen gel

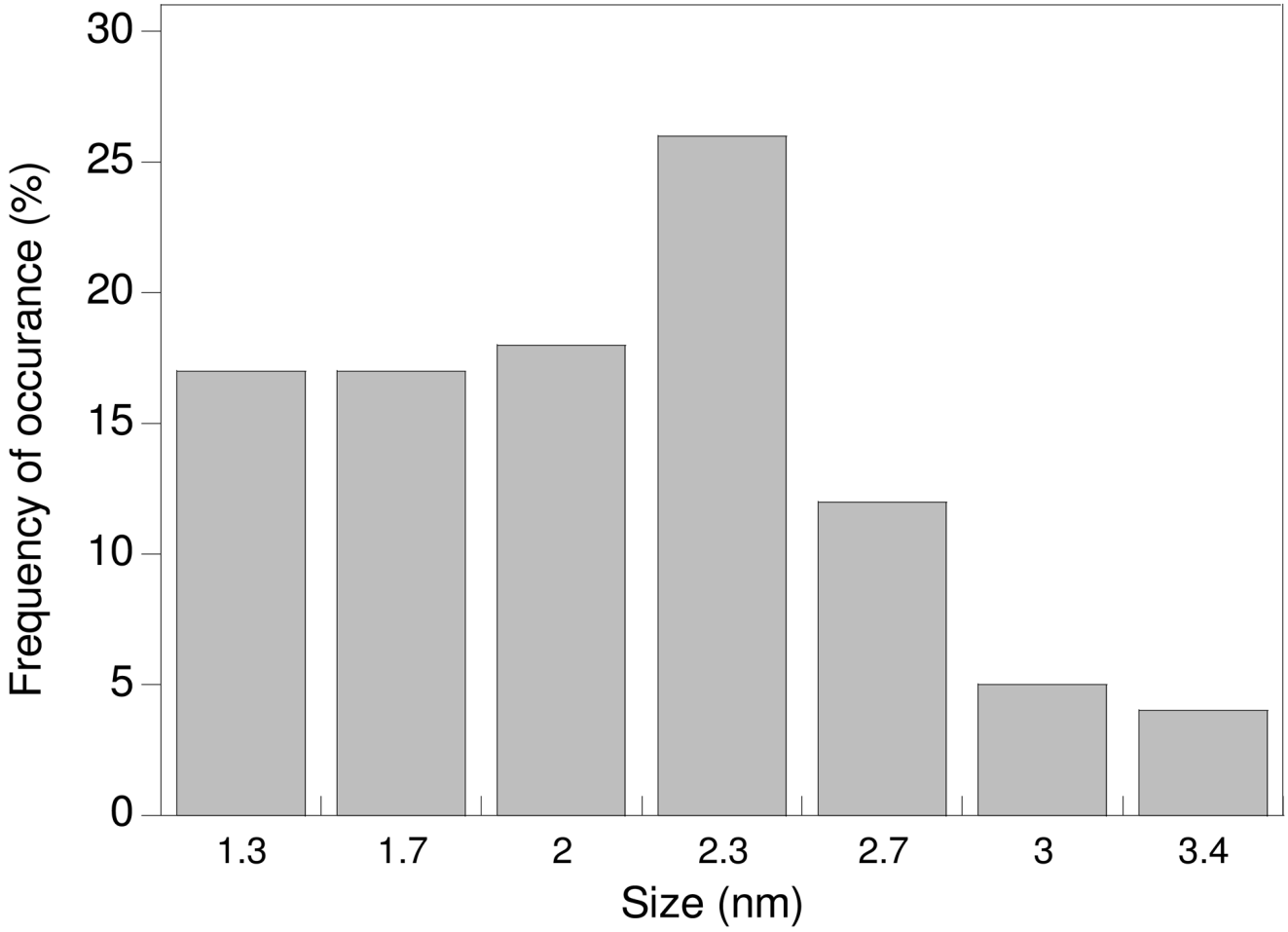

Figure 2.
Histogram of the nanoparticles sizes measure from TEM image (constructed by measuring sizes of 200 particles).

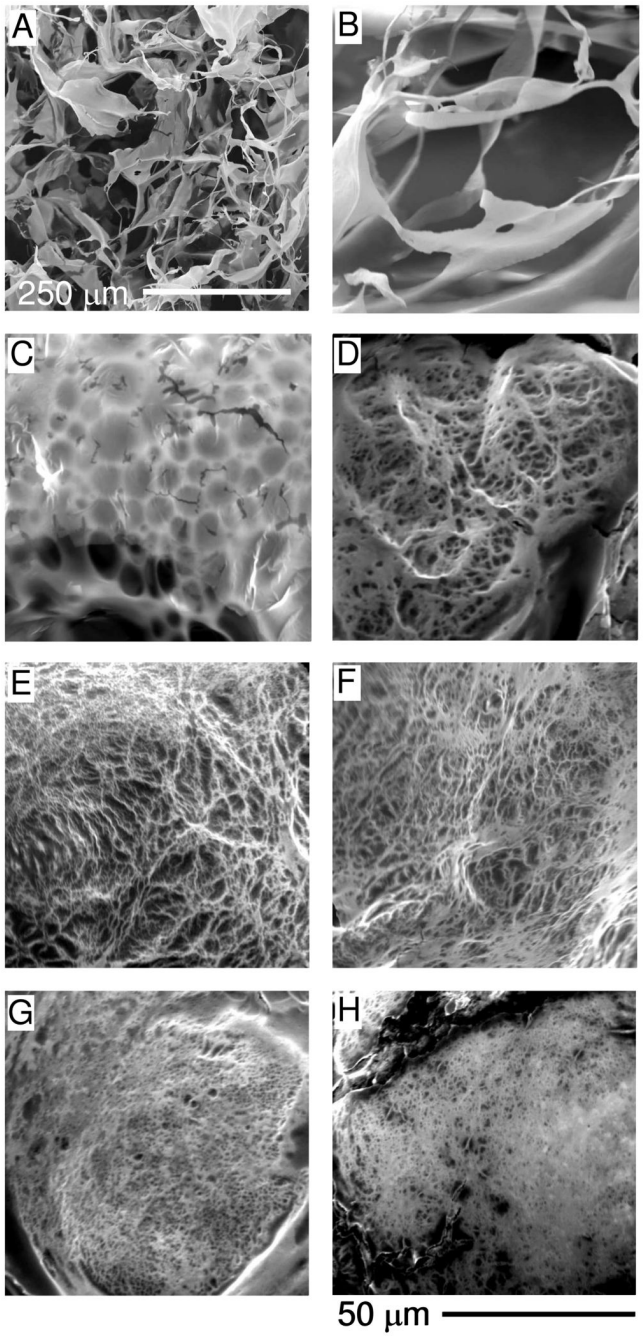

Figure 3.
SEM images of lyophilized collagen gels. Uncrosslinked collagen, Mag 219× (A), Mag 1400× (B), Collagen crosslinked with EDC (C), AU1 (D), AU2 (E), AU3 (F), AU4 (G), AU5 (H). All images (except A) were taken at 1400× Mag.

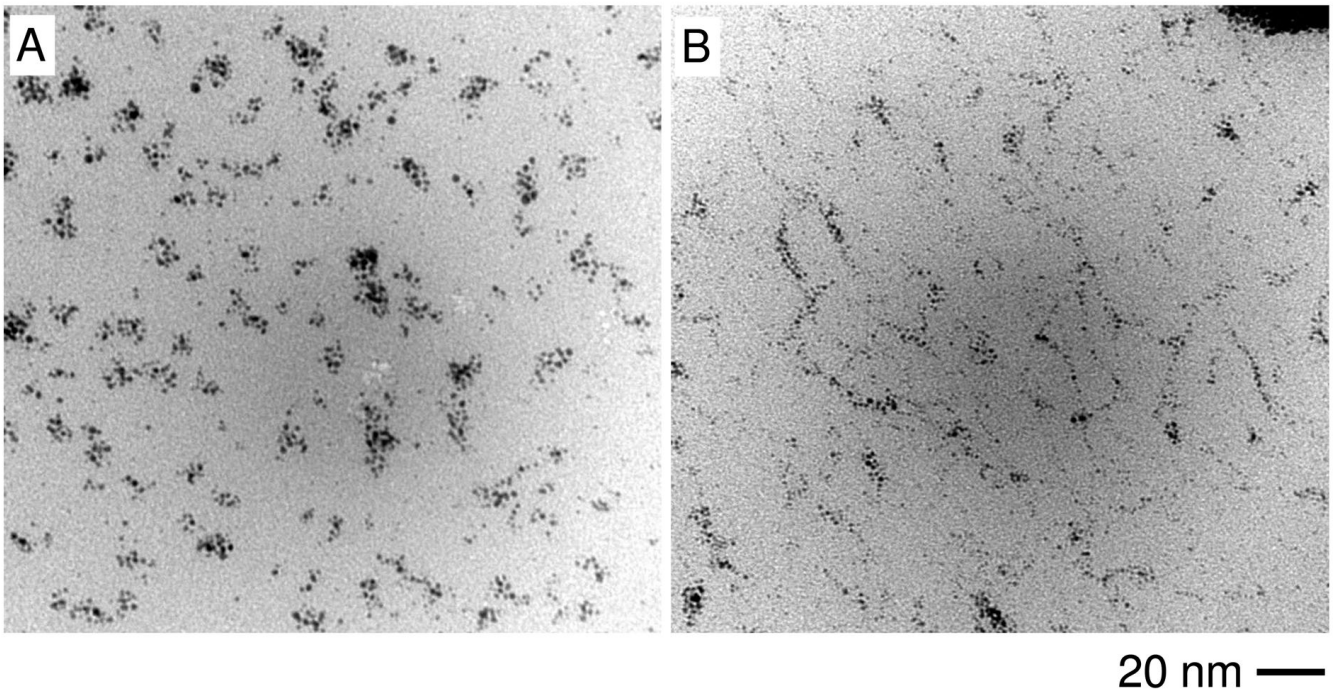

Figure 4.
TEM images of lyophilized collagen gels preserved in the Araldite resin. AU1 (A) and AU5 (B).

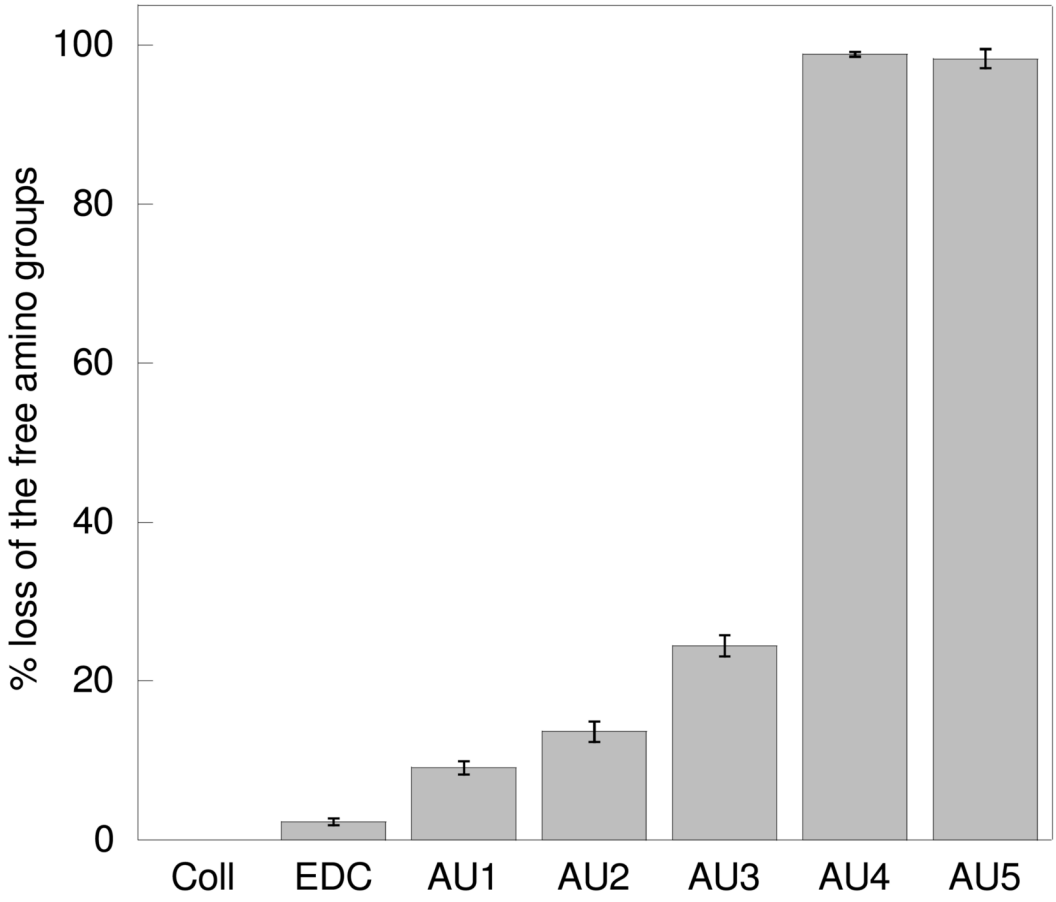

Figure 5.
Histogram of the % loss of free amino groups normalized with respect to uncrosslinked collagen determines by TNBS assay. The error bars reflect standard deviation calculated from 6 samples.

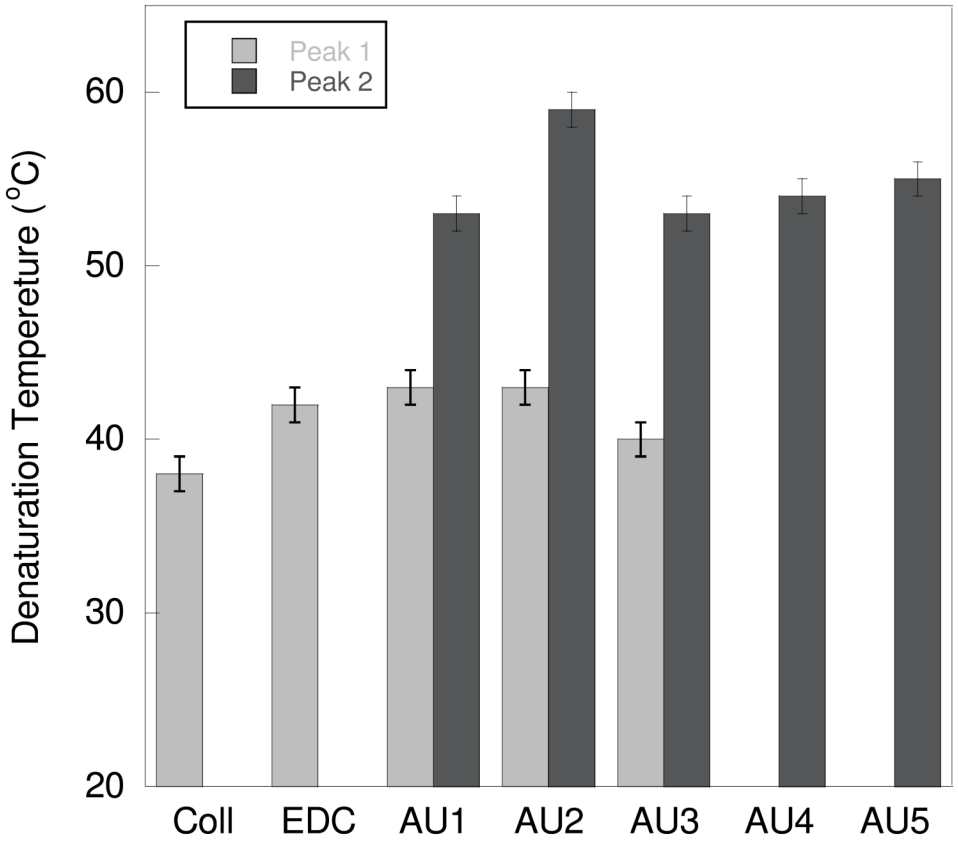

Figure 6.
Histogram of shrinkage temperatures recorded with micro-DSC. The error bars reflect standard deviation calculated from 3 samples.

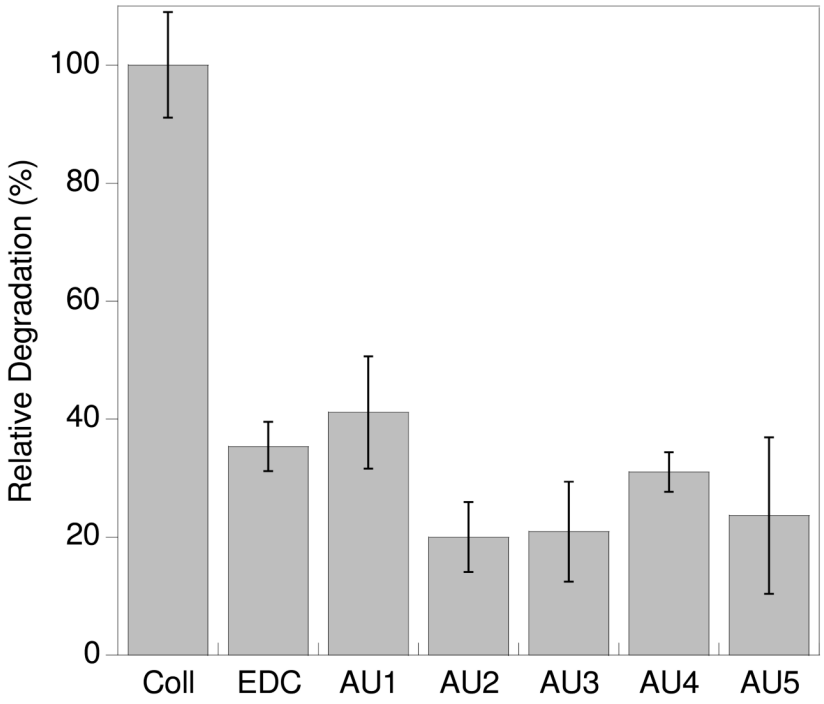

Figure 7.
Histogram of collagenase degradation assay normalized with respect to uncrosslinked collagen. The error bars reflect standard deviation calculated from 6 samples.

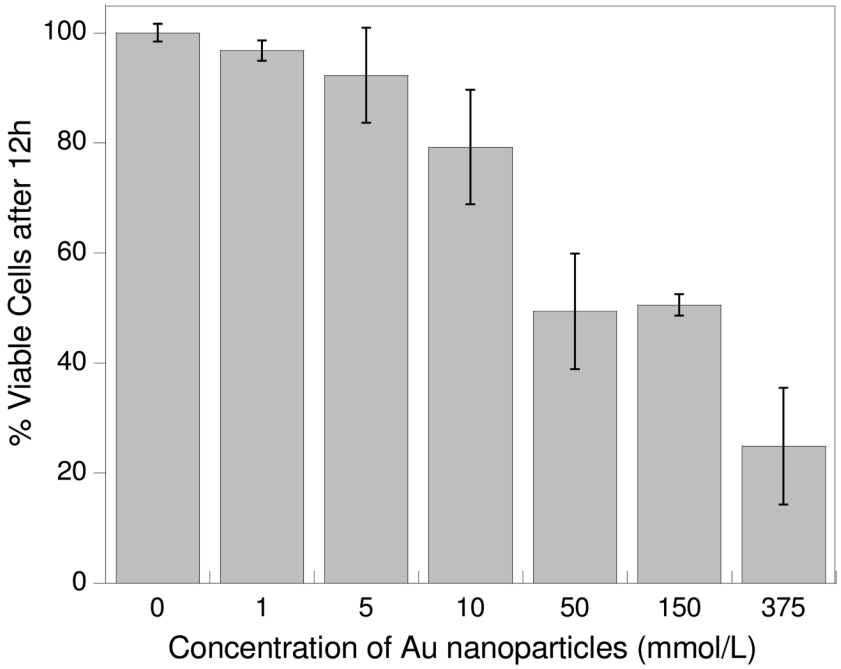

Figure 8.

Histogram of percentage of viable cells (3T3 fibroblasts) after 12 hours measured with CellTiter® Assay after addition of 2.4 nm Au-Tp clusters to the cell media, normalized with respect to control (DI water added). The error bars reflect standard deviation calculated from three repeats on two different plates.

Table 1

Collagen gels composition.

Sample Name	Mass Ratio (Coll:TPAu)	Molar Ratio (Coll:TPAu)
Coll	1 : 0	1 : 0
Coll/EDC	1 : 0	1 : 0
AU1	5 : 1	1.4 : 1
AU2	3.3 : 1	1 : 1.1
AU3	1.7 : 1	1 : 2.2
AU4	1 : 1.2	1 : 4.4
AU5	1 : 1.8	1 : 6.7