Expert Opinion

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Collagen-coated microparticles in drug delivery

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Advantages of drug-incorporated collagen particles have been described for the controlled delivery system for therapeutic actions. The attractiveness of collagen lies in its low immunogenicity and high biocompatibility. It is also recognized by the body as a natural constituent rather than a foreign body. Our research and development efforts are focused towards addressing some of the limitations of collagen, like the high viscosity of an aqueous phase, nondissolution in neutral pH buffers, thermal instability (denaturation) and biodegradability, to make it an ideal material for drug delivery with particular reference to microparticles. These limitations could be overcome by making collagen conjugates with other biomaterials or chemically modifying collagen monomer without affecting its triple helical conformation and maintaining its native properties. This article highlights collagen microparticles' present status as a carrier in drug delivery.

Keywords: collagen, controlled delivery, drug delivery, microparticles

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1. Introduction

Many of the present controlled release devices for *in vivo* delivery of macromolecular drugs involve elaborate preparations, often employing either harsh chemicals such as organic solvents or extreme conditions, like elevated temperatures. These conditions have the potential to destroy the activity of sensitive macromolecular drugs, such as proteins or polypeptides. In addition, many devices require surgical implantation and in some cases the matrix remains behind or must be surgically removed after the drug is exhausted. Use of microparticles loaded with macromolecular drugs avoid all these shortcomings when appropriately formulated.

Microparticles usually refer to spherical particles with diameters in the micron range. The size normally ranges from 0.1 µm to approximately 100 µm. The upper limit of 2 mm is also included as microparticles [1]. They represent a simple yet effective system for delivering drug to specific tissue sites *in vivo* and can be used to control the tissue response to injury at the site of implantation. Microparticles can have drugs incorporated in the core or dispersed throughout the polymer matrix for the development of controlled delivery systems.

Targeting delivery of drugs to the diseased lesion is one of the most important aspects of drug delivery devices. Microparticle carriers have important applications for the administration of therapeutic molecules. Moreover, microparticles have loading capacity for drugs or growth factors and for tissue regeneration as injectable scaffolds. Cell culture on microparticles may produce a large number of cells during a relatively short period. The trypsinization procedure, which impacts the cells, can be avoided since the expansion of cells can be achieved by the simple addition of new microparticles to culture medium. It is also more convenient if the cell-attached microparticles are used directly as injectable cell microcarriers [2].

Microparticles have been used for site-specific, controlled delivery of a wide variety of bioactive compounds like antineoplastic drugs, narcotics, anesthetic

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agents, proteins, DNA and growth factors for a range of applications including chemotherapy, cardiovascular disease, hormone therapy and vaccine development [3].

When used *in vitro*, biodegradable microparticles are specially appreciated since traditional cell microcarriers are less or even non-absorbable. Moreover, biodegradable carriers for drug delivery have several advantages over the others as they are biocompatible and need not be surgically removed after the delivery of the drug [4]. Collagen is an interesting natural material for the preparation of microparticles. The attractiveness of collagen rests largely on the view that it is a natural material of low immunogenicity and is therefore seen by the body as a normal constituent rather than a foreign matter [4]. Collagen exhibits an extremely high biocompatibility with low antigenicity. It is also biodegradable and bioresorbable [5,6]. Today, it is considered as one of the most useful biomaterials in the realm of drug-delivery applications. It is a versatile material whose degree of crosslinking can be manipulated, thereby dictating the rate of drug release. Collagen has been used in the preparation of injectable hydrogel matrices, biocompatible implants (Zyderm® Collagen, INAMED Corp., California, USA), carrier systems for ocular applications, and flexible fleeces for the treatment of wounds (Opragen®, Lohmann, Germany) [7,8].

Collagen does not show any response when injected in most patients. But in some cases, it may have a positive or undersea (i.e., clinically negative but immunogenically positive) response. The augmented antigenicity might be enough to cause allergic reaction in patients who had no response to collagen implant.

2. Collagen microparticles: present status and their preparations

Owing to their excellent biocompatibility, the biodegradable polymers polylactic acid (PLA) and polylactic-co-glycolic acid (PLGA), including collagen, are the most frequently used biomaterials for microencapsulation of therapeutics and antigens [9-19]. *In vitro* and *in vivo* studies of these polymers make them a better candidate for developing microparticle composites with collagen. Both polycaprolactone (PCL) and PLGA are used in humans for the parenteral dosage form. *In vitro* and *in vivo* PCL and PLGA nanoparticles [20] loaded with heparin showed significant increase in antifactor Xa activity and aPTT (activated partial thromboplastin time), confirming the oral absorption of heparin released from microparticles exhibiting their absolute bioavailability in rabbits. Similarly, biodistribution of PCL and PLGA nanoparticles radiolabeled with Tc-99m showed enhanced bioavailability for etoposide and reduced the associated toxicity [21]. Nicoleta *et al.* [22] carried out histological analysis of dexamethasone containing PLGA superparamagnetic microparticles for the local treatment of arthritis and found no inflammatory reaction in the joint. This type of carrier could represent a suitable magnetically retainable, intra-articular drug delivery system for treating joint diseases such as arthritis or osteoarthritis. Se *et al.* [23] used PCL microparticle-dispersed PLGA solution as a potential injectable urethral bulking agent in hairless mouse (subcutaneously). They observed gradual infiltration of surrounding tissue, including blood vessel, into the implant for up to 8 weeks without the initial volume change and with little inflammatory response, thus showing that the solution is a good candidate as an injectable bulking agent for the treatment of urinary incontinence.

Collagen/PLGA microparticle composites for local controlled delivery of gentamicin have been prepared by Monika Schlapp *et al.* [24]. In that paper, the positive effect of collagen on tissue regeneration and local delivery of low molecular weight compounds for an extended time are described. Collagen, including other natural biomolecules have domains in their molecules recognized as ligands that can specifically bind to integrin on cell membrane, and thus can effectively accelerate cell attachment and spread. PLA microparticles with a larger amount of collagen on their surfaces have been successfully fabricated by a method of aminolysis and grafting – coating for *in vitro* chondrocyte culture, showing better attachment, proliferation, viability and distribution on the microparticles immobilized with a larger amount of collagen. These results determine that the collagen-coated PLA microparticles could effectively support the attachment and proliferation of chondrocytes. PLA is nontoxic and can be processed easily. However, its cytocompatibility needs to be improved because its hydrophobicity retards cell attachment [25,26]. One approach to improve its cytocompatibility is to introduce collagen onto the surface of PLA through surface modification because collagen can specifically bind with integrins on cell membrane, which can effectively accelerate cell attachment and spreading [2].

Xiao *et al.* [12] prepared PMMA (polymethyl methacrylate)-PLA microparticles coated with collagen. They introduced PMMA onto the PLA surface using a grafting polymeric method. The PLA particles were photooxidized by immersing them in H_2O_2 and UV irradiated for 50 min. The excess was removed by rinsing with deionized water. The particles were then immersed in methyl meth acrylate (MMA), (15%) and the grafted particles were obtained by graft polymerization under UV for 30 min. These were then rinsed with deionized water at 65°C to remove homopolymers, and dried. Collagen in acetic acid solution was dropped into PMAA-PLA microparticle suspension to coat them. They were finally washed with deionized water and dried at room temperature. More study on the cytocompatibility *in vitro* and injectability *in vivo* of microparticles is needed.

Spherical collagen particles can be prepared using a spray-drying technique. Since viscosity of collagen preparations is high, only dilute solution of collagen has to be sprayed, which leads to thin and fragile hollow spheres. In our own study, we have coated collagen on PCL microparticles to increase its drug-loading capacity and facilitate cell attachment. The PCL

microparticles were prepared with PVA (polyvinyl alcohol) as a copolymer. Experiments were carried out to see if the same polymer with different copolymer ratio had any effect on the size and quality of the microparticles formed. PVA concentration in the external water phase has a great impact on the size of resultant microparticles. Use of 4% PVA in the external water phase resulted in uniformly sized (~ 1000 nm) microparticles. PCL has many advantages such as nontoxicity, processibility and biodegradability, but it is hydrophobic, and the encapsulation of doxycycline hydrochloride (DH) in the PCL microparticle was found to be very low. Hence, a surface treatment with collagen was required to improve the microparticle's drug-holding capacity [2,27]. Similar particles were prepared by Yi Hong *et al*. for use as chondrocyte carriers [2].

Berthold *et al*. prepared collagen microparticles using the following procedure. Collagen dispersion was emulsified in liquid paraffin containing sorbitan monolaurate 20 (Span® 20) using a homogenizer. Glutaraldehyde was added to crosslink collagen. Hydrogen peroxide solution was added to interrupt the crosslinking and the emulsion was then diluted with 2-propanol and centrifuged. The collagen microparticles were then purified by centrifugation, washed and dried (**Figure 1**) [5].

Collagen microparticles of diameters ranging from 3 µm to 40 µm were prepared by Rossler *et al*. by emulsifying and crosslinking native collagen. Controlled denaturation of collagen was carried out to control the size of the microparticles. These could be used as carriers for lipophilic drugs, for example retinol, tretinoin, or tetracaine and lidocaine in free base form [28]. Marine sponge collagen has also been used for the preparation of collagen microparticles by Rössler *et al*. [29]. Collagen was extracted from marine sponge and particles prepared following the procedure used by D Swatschek [30].

Collagen microparticles (CMPs) were manufactured by Rossler *et al*. [29] with the objective to enhance the dermal delivery of all-trans retinol. The retinol was adsorbed from marine sponge onto the surface of CMPs. The remarkable result they found was that the presence of CMPs led to a faster and higher transport of retinol into the rat skin than the freshly precipitated drug [28]. This process offers an alternative and attractive source of collagen other than calf or bovine origin. Today biomaterial preparations from both the origins bear the risk of bovine spongyform encephalitis (BSE) and is easily transmitted to humans [31-34]. In order to improve the physical properties, atelocollagen was incorporated as an additive in microparticles fabricated by gelation of sulphadiazine-loaded hyaluran solution with calcium chloride through either a granulation or encapsulation process. Hyaluran, a natural glycosaminoglycan, in extracellular matrix, an effective biocompatible material, has been used as a carrier system for antibiotics in the microparticles. Here, incorporation of collagen had a more compact surface than particles with hyaluran alone. Collagen-modified hyaluran microparticles have potential as a release-rate-controlling material for crystalline drug such as sulphadiazine. Collagen, when combined with hyaluran, restricts the rapid solubility of hyaluran owing to the formation of a collagen fiber network [35].

A new antibacterial dressing has been developed from succinylated bovine collagen for infected wounds. The dressing contains an antibiotic, mupirocin, for both immediate and time-regulated release for controlling the infection, as the infected open wounds need special care. Succinylation offered advantages like better adherence of multicomposite dressing to the wound surface, anionic collagen network at neutral pH and sharp isoionic pH. These properties are not available with unmodified collagen and can advantageously be used for the delivery of drug from the multicomposite dressing systems.

The dressing consists of a sponge and a film, both prepared from succinylated bovine collagen. The sponge has a smooth ventral (dermal) surface, which forms the trilayer system by diffusion. The drug stays in the trilayer system because of physical entrapment, but starts releasing when it comes in to contact with aqueous fluid or when the wound exudates.

Release of the drug is immediate and sustained, regulated by differential physical entrapments between the free drug, drug-oaded microparticles and scaffold. The antibiotic was released slowly from the dressing in *in vitro* experiments for 15 days. Antibacterial activity of the dressing was tested on an *in vitro* model by agar diffusion and *in vivo* using a rat wound model experimentally infected with *Staphylococcus aures*. The dressing containing mupirocin reduced the number of living bacterial cells in the infected tissue to almost zero during the course of observation [36].

At elevated temperatures, collagen particles are denatured and degraded. To preserve the native structures of collagen, preparations can be sprayed into liquid nitrogen. The particles are subsequently frozen, tempered, lyophilized, crosslinked and sterilized [37,38].

3. Chemical modification of collagen

The limitations of collagen (thermal instability (denaturation), high viscosity of collagen in aqueous phase, biodegradability, nondissolution in neutral pH buffer etc.) can be addressed and overcome by collagen conjugates. Collagen itself or its composites with other biomaterials including chemically modified collagen varieties can be used to overcome the above difficulties.

The solubilization property, stability, viscosity and biodegradability are factors that can be altered by chemical modification of collagen. Collagen has charge groups such as carboxyl and amino groups on the side chains of the triple helical structure of amino acids' three alpha chains. These amino acids include lysine, arginine, aspartic and glutamic acids. Polar groups of these amino acids lie out of the coiled-coil structure of collagen monomer. Chemical modification of one of these polar groups offer properties like sharp isoionic pH, solubility at neutral pH under

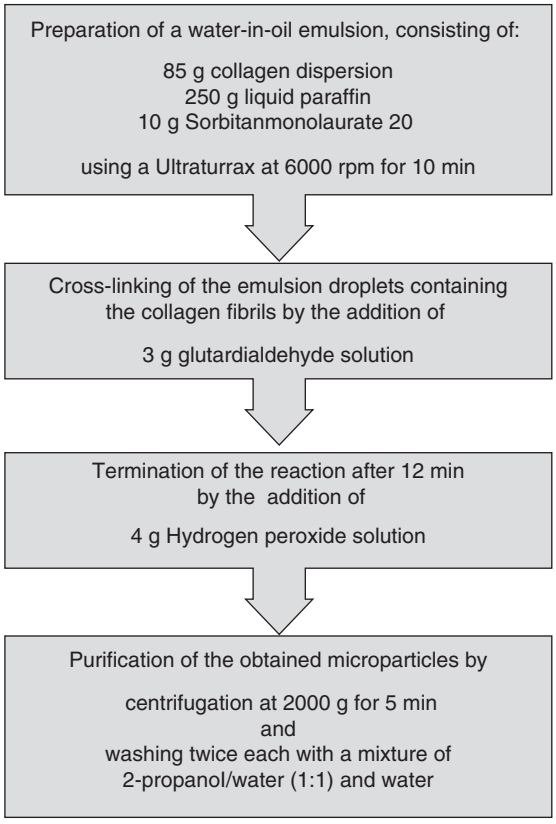

Figure 1. Schematic diagram for the preparation of collagen microparticles.

Source: Berthold A, Cremer K, Kreuter J. *Eur J Pharm. Biopharm* 1998;45:23-9 [5].

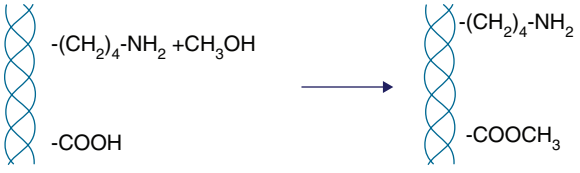

Figure 2. Methylation.

physiological conditions, control of viscosity, resistance towards degradation etc. It will also facilitate formation of collagen conjugates with other biomaterials. All these properties can be used advantageously to form microparticles and loading of drugs and other biomolecules. The net charge on native collagen monomer is almost zero because of Zwitter ion. This is due to a moderate number of positively and negatively charged groups. These groups are derived from the diamino and dicarboxylic acids built into the collagen monomer. In the isoelectric state and in equilibrium with aqueous buffers in the pH range 5 – 8, these polar groups are completely ionized. Collagen is an amphoteric, ionic structure attaining the highest degree of charge, both positive and negative, in the isoelectric pH range. These charged centers of the proteins and Zwitter ion exist in balance with each other. It seems that this balance can be disturbed only by some mechanism that is able to suppress either the positive or negative charges. The transfer of proton from one position of binding to another will depend on the pH value of the solution as the following simplified equations bring out.

$$COO^{-}R.NH_3^{+} + H^{+} \rightarrow COOHR.NH_3^{+} \text{ (in acid solution)}$$

$$COO^{-}R.NH_3^{+} - H^{+} \rightarrow COO^{-}RNH_2 \text{ (in alkaline solution)}$$

This dipolar partnership in native collagen molecule can be broken by chemical modification of the side chains of polar amino acid groups of either lysine, arginine, glutamic and aspartic acids. The net charge of native collagen (without chemical modification) is almost zero at physiologic pH. However, chemically modified collagen can have a net negative or positive charge, based on which oppositely charged drugs can be linked to it. The ionic interaction between drug and modified collagen prevents rapid release of the drug from the device.

Chemical modification of the amino groups may be performed by acylation with acetic anhydride or other anhydride such as succinic anhydride. Esterification of carboxyl groups may be carried out by standard reaction with acidified alcohol, preferably a water-soluble aliphatic alcohol such as methanol, ethanol etc. Succinylated collagen has a large negative net charge at physiologic pH and methylated collagen a large positive net charge.

3.1 Methylation of collagen

Collagen as gel or powder was methylated in the acidified methanol (acidified methanol containing 0.1 M HCl was dehydrated by intermittent stirring with excess anhydrous Na_2SO_4) for a period of about 7 days at room temperature in a tightly sealed vessel. After methylation, the collagen product was dried in vacuum and pulverized with a 100-mesh sieve (**Figure 2**).

3.2 Guanidination of collagen

One hundred grams of dry fiber collagen was suspended in 1 liter of water and the pH adjusted to 9.5 by the addition of NaOH. Eighty grams of 1-guanyl-3, 5-dimethylpyrazole nitrate were dissolved in 1 liter of water and the pH again adjusted to about 9.5. The collagen dispersion and the reagent were mixed and allowed to stand for 7 days at about 4°C with intermittent stirring. The collagen quaternary structure remained stable during the reaction. After guanidination the collagen was washed with water and collected by centrifugation (**Figure 3**) [39,40].

3.3 Succinylation of collagen

Modification of lysine with dicarboxylic anhydrides such as succinic anhydride prevents the subsequent cleavage of lysyl peptide bonds with trypsin. In addition, modified proteins act as better substrates for proteases. Succinic anhydride reacts with the ε-amino group of lysine and the amino-N-terminal

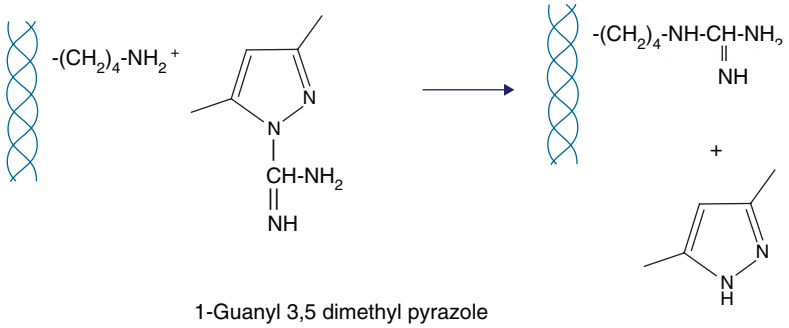

Figure 3. Guanidination.

α-amino group of proteins, in their nonprotonated forms, converting them from basic to acidic groups. Thus, one effect of succinylation is to alter the net charge of the protein by up to two charge units.

Two grams of collagen were dissolved in a mixed solution of 1.6 ml of concentrated hydrochloric acid and 100 ml of water, to which a 5-N aqueous solution of sodium hydroxide was gradually added to adjust the pH to 13 while stirring. A 0.07-g quantity of succinic anhydride (corresponding to 1 mol per 1 mol of the ε-amino group at the side chain of collagen) was dissolved in 10 ml of acetone, and the whole quantity of the acetone solution was gradually added to the above described collagen solution, followed by stirring for 1 night while controlling constantly the pH to 13 with a 5-N aqueous solution of sodium hydroxide. The precipitate was separated by filtration, washed adequately with water and dried to obtain 2 g of succinylated collagen. The modification ratio of the ε-amino group of the collagen was 30% (**Figure 4**) [39].

3.4 Acetylation of collagen

Acetylation of collagen alters the isoelectric point of collagen to 4.2. Acetylated collagen behaves as an anion at physiological pH (7.2) and remains in solution (colloidal state). Pure collagen (1 mg) was taken in 100 ml of deionized water at pH 3.0. After collagen solubilization, the pH was adjusted to 9.0 by adding 1 M NaOH solution. Eighty moles of acetic anhydride was mixed with acetone and gradually added to collagen suspension. During addition, the pH was maintained at 9.0 by adding NaOH solution. Acetylated collagen was precipitated by bringing down the pH to 4.2 using dilute HCl. The modification was carried out under cold condition by placing the beaker containing collagen solution on ice. After precipitation, the acetylated collagen was washed repeatedly in water at pH 4.2 and made to swell in 200 ml of Milli-Q water to form a uniform solution at pH 7.0 to get pure acetylated collagen (**Figure 5**) [27].

3.5 Desamidation

Achilles tendon, cleaned from adhering tissues and minced in a homogenizer was soaked in a nonionic detergent overnight and washed thoroughly with water, limed and delimed. The minced tissue was treated with sodium sulphate – sodium hydroxide solution for 7 days under cold room conditions (8°C). The swollen mass obtained by the above treatment was further homogenized and treated slowly with cold concentrated HCl; the temperature of the homogenized paste was not allowed to go above 20°C and the pH of the paste was brought down to 2.8. The precipitated mass obtained was dialyzed against distilled water three times. The collagen gel thus obtained forms a homogeneous solution in water, which is stored under frozen conditions (**Figure 6**).

4. Discussion

Today, collagen is being incorporated in a number of biomedical applications with expanded usage. Questions have been raised concerning the immunogenicity of the protein in humans. Collagen is an excellent biocompatible material and, owing to its low toxicity and poor immunogenic reactions, its antigenicity was considered nonexistent [41,42]. Today, concerns are raised over animal-derived collagen because of a massive immune response causing secondary effects such as damage of organs by immunocomplexes or cross-reactions of antibodies with human collagen. It may induce autoimmune diseases. In this context of the induction of antibody response, we need to differentiate between immunogenicity, the existence of antibody response and antigenicity. Animals and humans can produce antibodies to three antigenic determinants in the collagen molecule: the nonhelical telopeptide regions (P-determinants); the amino acid sequence in the helical section (central determinants); and the triple helix structure (helical determinants) [43,44]. The importance of each determinant depends on the nature of the processing of collagen products. Selective removal of telopeptides by enzyme treatment will suppress antigenicity owing to P-determinants [45,46]. Additional cross-linking with glutaraldehyde would reduce antigenicity further, but does not eliminate it altogether [47,48]. After these two treatments, the immunogenic response depends on the collagen source as well as the test technique and the species used for animal experiments [6]. Collagen immunological response

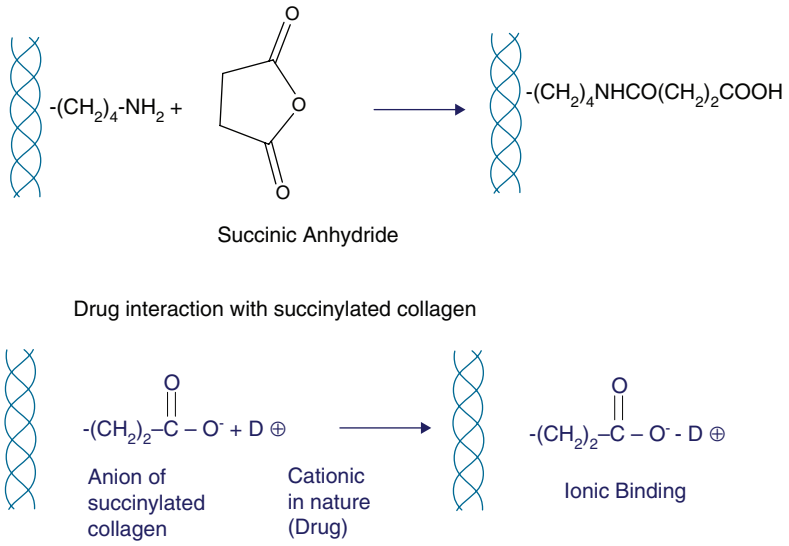

Figure 4. Succinylation.

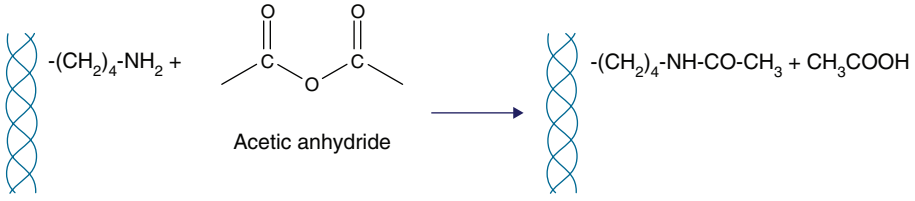

Figure 5. Acetylation.

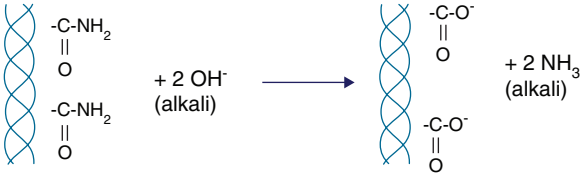

Figure 6. Desamidation.

has been studied by a number of researchers [43,47,49]. Other than P-determinants only 3% of patients had elevated anti-implant antibody levels against both the helical conformation and the primary sequence after first injection and experienced localized inflammatory response resulting in erythemas, swelling, pruritus and induration without any general systemic complaints. Another 2% showed reaction to a second injection. Details of similar studies are described for the commercial product Zyderm, which is pepsin-solubilized bovine collagen implant for tissue augmentation. In spite of all these concerns, collagen is considered safe [50] and only mildly antigenic, making it suitable for use as an injectable biomaterial.

Here the use of microparticles of collagen system could simplify the use by injection. Some of the limitations of collagen like the high viscosity of an aqueous phase, nondissolution in neutral pH buffers, biodegradability and thermal instability can be addressed by chemical modifications or surface coating of microparticles with collagen [29,30] or its composites with other biomaterials.

5. Expert opinion

5.1 Current status of topic under discussion

In spite of the limitations of collagen, such as thermal denaturation, high viscosity in aqueous phase etc., microparticle preparations have been reported from this protein by a few researchers. Xiao *et al.* [12] prepared collagen microparticles by dispersing the protein in liquid paraffin. Rossler *et al.* [29] used marine sponge as a source of collagen and found microparticles transported faster and a higher amount of drug into rat skin. Maffia *et al.* [37] spread liquid particles to preserve the microparticle structure of collagen.

Other workers have reported preparation of collagen microparticles making a composite of this molecule with PLGA, PCL and PMMA-PLA. These composites offer

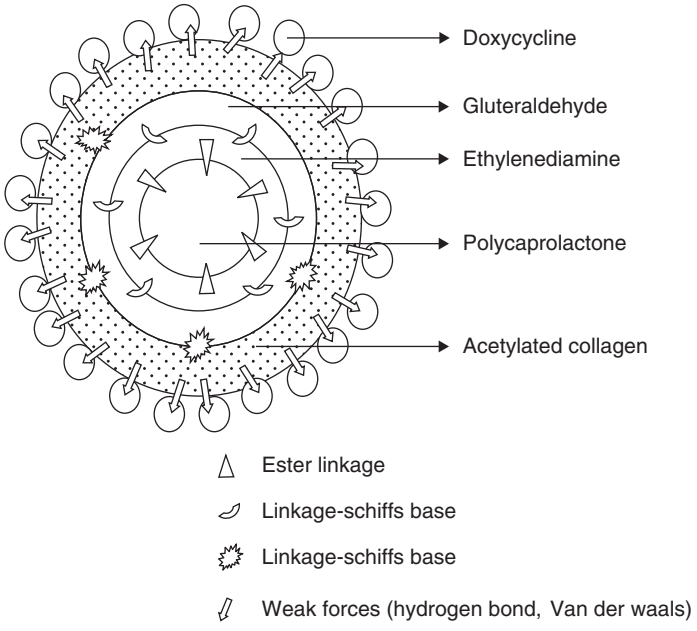

Figure 7. Schematic diagram of collagen-coated PCL microparticle.

excellent biocompatibility, compact surface and higher drug-loading capacity and effectively support the attachment and proliferation of cells.

In another very interesting study, a succinylated collagen trilayer system comprising microparticles has been used effectively for the release of antibiotic. This trilayer system facilitates controlled release of drug for 15 days. In our own study, collagen-coated PCL microparticle could deliver the drug in a controlled fashion for a period of > 10 days (**Figure** 7).

Denaturation temperature of collagen is 37°C. Above this temperature, collagen loses its secondary and tertiary conformation, and renaturation becomes difficult if there is considerable delay in microparticle preparation. Further renaturation of collagen is never 100%, even if it is done in the shortest possible time. Owing to this property, the yield of microparticles is considerably less compared with gelatin, alginate, chitosan or other biodegradable polymer microparticles. The second drawback of high viscosity is its aqueous phase, which limits microparticle preparation, but this can be circumvented by pH adjustment and use of miscible organic solvents that do not alter the properties of collagen.

Collagen biodegradability can be controlled and kept under check if collagen composite with other biodegradable polymers are formed. Chemical modification on the side chains of polar amino acid residue will also offer enhanced thermal stability and resistance to degradation.

5.2 Status in the next 5 – 10 years

Today, if we look into the current status of microparticles of collagen in drug delivery, we find that only a few laboratories are working on it. Collagen drug-delivery devices have immense advantages as they are recogonized by the body as a natural constituent rather than a foreign body on account of its low immunogenicity and high biocompatibility. Many laboratories the world over have reported drug-delivery devices based on this protein. The state of the art in controlled delivery devices in collagen is well established and it is only a matter of time before more researchers take interest in the preparation of collagen-coated microparticles for drug delivery to offer improved systems.

5.3 How this will be achieved

Unlike earlier, knowledge of collagen preparation, modifications and structure is available in the open domain. Sourcing collagen from same species is the best option for preparing microparticles. Very soon, many labs around the world should start to source certified human tissues like placenta, amnion etc. for collagen preparations. Once this is done, no other biopolymer would be able to compete with collagen and this would lead to more labs reporting microparticle delivery systems using collagen.

Declaration of interest

This review article deals with the present status of microparticles in various drug delivery formulations. The opinion expressed here are the personal opinions of the authors based on the available literature. It does not bind the authors in any way. The authors state no conflicts of interest and have received no payment in the preparation of this manuscript.

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