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Controlled self assembly of collagen nanoparticle

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Abstract In recent years carrier-mediated drug delivery has emerged as a powerful methodology for the treatment of various pathologies. The therapeutic index of traditional and novel drugs is enhanced via the increase of specificity due to targeting of drugs to a particular tissue, cell or intracellular compartment, the control over release kinetics, the protection of the active agent, or a combination of the above. Collagen is an important biomaterial in medical applications and ideal as protein-based drug delivery platform due to its special characteristics, such as biocompatibility, low toxicity, biodegradability, and weak antigenicity. While some many attempts have been made, further work is needed to produce fully biocompatible collagen hydrogels of desired size and able to release drugs on a specific target. In this article we propose a novel method to obtain spherical particles made of polymerized collagen surrounded by DMPC liposomes. The liposomes allow to control both the particles dimension and the gelling environment during the collagen polymerization. Furthermore, an optical based method to visualize and quantify each step of the proposed protocol is detailed and discussed.

Introduction

Biodistribution and pharmacokinetics are the major factors that directly influence and determine the efficacy of a drug treatment. Conventional or free drugs upon administration are widely distributed throughout the body and can adversely affect other cells than the targeted treatment area. Moreover, drugs also are often cleared too quickly from the body ushering in a treatment approach of higher doses, or continuous infusion. Finally, pharmaceutical compounds alone may be unstable and degrade quickly. In these last years, to overcome all these limitations, several Drug Delivery Systems (DDS) have been designed by using natural drug carriers (Vinogradov et al. 2006; Maham et al. 2009).

A wide variety of proteins have been used and characterized for DDS, including the ferritin/apoferritin protein, the small heat shock protein, albumin, soy, and whey protein.

The most recent nanomedicine development has been focused upon the protein-based platform of

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naturally self-assembled protein subunits. In this context, collagen, the major component of the extracellular matrix and largely present in the mammalian body, has been one of the most newly developed biomaterial for drug delivery and therapeutic applications since it forms hydrogels without requiring the use of chemical crosslinking in its preparations, for its multifunctionality and biodegradability. Classical methods for particle production often lead to instable matrix, or complicated/drastic steps for preparation such us UV radiation or chemical treatments and thus, innovative methods seem to be necessary to render this self-assembled protein ready available for drug delivery (Soppimath et al. 2001; Lee et al. 2001; Wallace and Rosenblatt 2003).

In this article, we propose a novel method for preparing a collagen based drug delivery platform by using lipid vesicles cages as a mould to produce spherical particles of controlled size. Since the physical properties of a collagen hydrogel (i.e., mesh size) can be easily controlled, a fine tuning in the extent of the drug's release is allowed (Gelman et al. 1979; Sano et al. 2003).

Materials and methods

Materials and laurdan labeling

Laurdan labeling was performed directly in the multilamellar vesicles (MLV) sample. 1 μL of Laurdan (Sigma) stock solution was added per milliliter of MLV. Laurdan was stocked at a concentration of 1 mM in dimethyl sulfoxide (Me$_2$SO; Sigma, St. Louis, MO), and it was renewed every 3 weeks. After 30 min of incubation in the dark at 28 °C, 200 mL of MLV were mounted upon a microscope slide.

Two-photon confocal microscopy

Laurdan, auto-florescence, and second harmonic generation intensity images have been obtained by using an inverted confocal microscope (DMIRE2, Leica Microsystems, Germany) using a 63× oil immersion objective (NA 1.4) with a mode-locked titanium–sapphire laser (Chamaleon, Coherent, Santa Clara, CA). Internal photon multiplier tubes collected

images in an 8 bit, unsigned images at a 400 Hz scan speed.

Maps of the fluorescence intensity of Laurdan, excited at a wavelength of 800 nm and collected in the range of 400–600 nm, were recorded. The collagen autofluorescence was stimulated by exciting the samples at 750 nm. Autofluorescence maps were recorded by collecting the emitted photons in the range of 400–550 nm. Second harmonic generation (SHG) has been stimulated from the oriented collagen fibrils by using an excitation radiation wavelength of 800 or 850 nm and the emission was collected at 400 or 425 nm, respectively. All the experiments were performed at 37 °C.

Atomic force microscopy

AFM imaging was performed using an SPMagic SX Atomic Force Microscope (Elbatech, Italy) in contact operation mode by using a constant probe force of 0.5 nN. Samples were kept in an aqueous environment throughout the measurement procedure. The microscope probe consisted of an ultra-sharp silicon nitride cantilever of nominal force constant $k = 0.05$ N/m with a tip radius of less than 10 nm (MikroMash).This approach was aimed to minimize perturbations and artifacts due to salt crystallization following dehydration. Image analysis was performed using a WSxM software (Nanotec Electronica S.L., Spain). Samples were laid down on a glass coverslip. All images were flattened with a zero- or first-order polynomial fit, and for each image an appropriate threshold value of the contrast was set to discriminate between objects and background.

Dynamic light scattering

Dynamic light scattering measurements was performed using a commercial light scattering set-up ALV spectrometer (ALV, Langen, Germany) consisting of a CGS-5000 rotating arm goniometer, a photomultiplier tube (PMT) (EMI, Ruislip, UK), an ALV 5000 multi-tau digital correlator operating with a sampling time of 200 ns, and an Innova 70 argon ion laser (Coherent, Santa Clara, CA) operated at 488 nm and 100 mW. The scattering cell was immersed in a refractive index matching fluid (toluene).

Dynamic light scattering (DLS) technique measures the intensity autocorrelation function $g_2(\tau)$, where τ is the lag time. The $g_2(\tau)$ can be related to the field autocorrelation function $g_1(\tau)$ through the Siegert relation $g_2(\tau) = 1 + \beta g_1^2(\tau)$, where β is an instrumental constant equal to one in our setup (Papi et al. 2005).

The mathematical form of $g_1(\tau)$ depends on the physical properties of the system investigated. For a monodisperse solution of noninteracting particles, a single exponential function with decay time τ is obtained. For a polydisperse sample, $g_1(\tau)$ is no longer a single exponential. In this case, the distribution of decay rates on $g_1(\tau)$ can be taken into account by introducing a weighting function: $g_1(\tau) = \int_0^\infty p_I(r)e^{-\Gamma(R_h)\tau}dr$ where $p_I(r)dr$ is the intensity-weighted radius distribution function, describing the distribution of the fraction, in the interval dr, of the intensity scattered by a particle of hydrodynamic radius R_h and decay rates $\Gamma(R_h) = kTq^2/6\pi\eta R_h$, with η the water viscosity and k the Boltzmann constant, T the temperature and q the scattering wavevector given by $q = (4\pi n/\lambda)\sin(\theta/2)$ where θ is the scattering angle, λ the laser wavelength, and n the solvent refractive index. The value $p_I(r)$ can be obtained using the regularized Laplace inversion of the intensity autocorrelation function (CONTIN). In this case the intensity-weighted radius distribution is obtained by a direct numerical inversion of the DLS data (De Spirito et al. 2006).

Results

To produce collagen hydrogel nanoparticle, we take advantage of classic procedures for liposome preparation (Torchilin 2005; Maulucci et al. 2005; Cevc and Marsh 1987). Indeed, this procedure consist first in the production of a dehydrated lipidic film, that is successively hydrated. The hydration allow the spontaneous formation of multilamellar vesicles.

The basic idea behind our approach consist in hydrate the lipidic film with a solution of gelling proteins. If the gelling time is much larger than the time required to produce liposome, protein gelation will occur inside the liposome. To include drugs (or any compound of interest (e.g., DNA, etc.) it is enough to add them in the gelling solution before the hydration.

Procedure for the collagen nanoparticles controlled polymerization

In Fig. 1 the whole procedure for the collagen nanoparticles preparation is schematically sketched in the six main steps. In detail:

Step 1–2 dissolution of lipids in chloroform and multilayer formation

10 mg of DMPC (Sigma) have been dissolved in 1 mL of chloroform in a glass test tube, the sample was vortexed for 2 min and let dry under nitrogen flux till a thin multilayer film is visible on the bottom of the test tube. The time request for a complete evaporation of chloroform depends on the temperature of the tube.

Step 3–4 hydration of the multilayer and multilayer vesicles formation

The hydration of the multilayer was performed with 1 mL solution of Type I Human Collagen (Cosmo Bio Co., Ltd) in PBS with a concentration of 0.1 mg/mL that have been previously heated at 28 °C. The hydration solution is then added in the test tube and the multilayer starts forming MLV. After the injection of the hydration buffer, the sample was kept at 28 °C for 1 h, during this time, every 20 min, the tube is vortexed for 5 min to facilitate vesicle formation. All these steps are realized at 28 °C because it was checked that at such temperature the collagen does not start to polymerize and because the DMPC is above its melting temperature.

Step 5 gelation of collagen stored in the MLV core

The sample has been diluted with 2 mL of PBS at 28 °C to avoid protein gelation outside the liposome cages. The polymerization of the collagen stored in the MLV core was achieved by heating the diluted sample for 2 h at 37 °C.

Step 6–7 nanoparticle releasing in solution and dialysis

To allow releasing of nanoparticles it is necessary to dissolve the lipid layer cages. For this purpose, a detergent, Triton ×100, is added to the sample with a
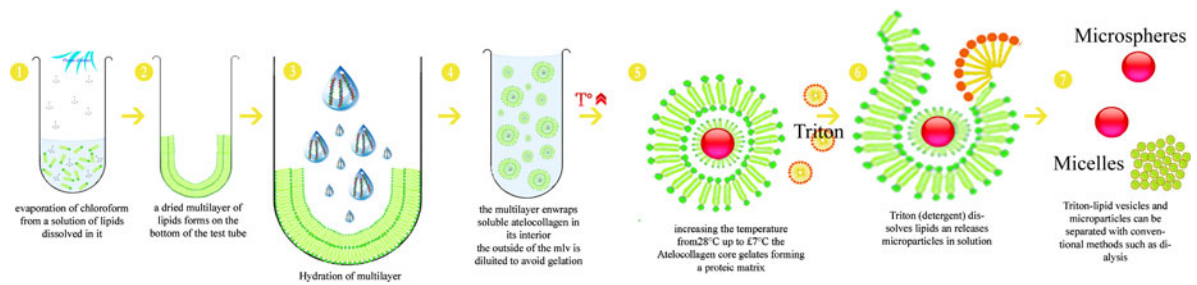
Fig. 1 Sketch of the collagen particles preparation. (1) Dissolution of lipids in chloroform. (2) multilayer formation. (3) Hydration of the multilayer. (4) multilayer vesicles formation. (5) Gelation of MLV core by increasing the temperature up to 37 °C. (6) Particle releasing in solution by adding Triton. See text for procedure details
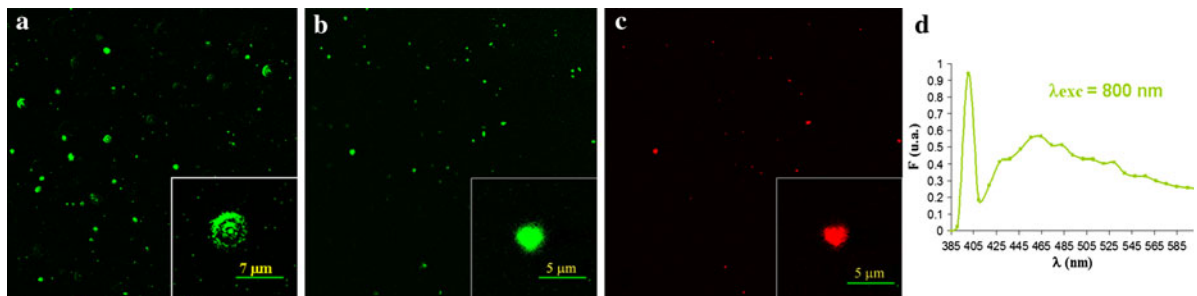

Fig. 2 Representative Laurdan two-photon intensity image of labeled DMPC multilayers liposomes (**a**). Representative autoflorescence intensity image of collagen molecules stored inside the DMPC multilayers liposomes (**b**). Representative SHG intensity image of self-assembled collagen matrix stored inside the DMPC multilayers liposomes (**c**). Collagen two-photon autoflorescence intensity and collagen polymers SHG intensity pick obtained by using an excitation wave length of 800 nm versus the emission wave length (**d**). All the images reported are 60×60 µm. In the *inset* a single particle magnification is also reported

30 mM final concentration. Finally, the solution was dialyzed against 0.1 M phosphate buffer to remove micelles.

Visualization and quantitative analysis of collagen nanoparticles polymerization by optical microscopy

Once step two of the protocol was performed, two-photon excitation microscopy images have been collected to establish the morphology of the DMPC liposomes and to quantify their size distribution (Parasassi et al. 1998). In Fig. 2a a representative laurdan intensity image is reported. This technique can resolve the liposome size in a wide scale range and eventually their internal structure and, its major advantage compared with other technique, is the possibility to quantify the variation of the liposome fluidity/rigidity with the variation of the physical–chemical conditions. Furthermore, laurdan molecules can be easily removed from the system, together with lipids, when liposome cages are dissolved and dialyzed at the last protocol step.

After the hydration with the collagen solution (step four), to check if liposome are filled by collagen molecules we performed auto-florescence two-photon excitation microscopy measurements (Gaus et al. 2003; Zoumi et al. 2002; Campagnola et al. 2002). In Fig. 2b a representative collagen's auto-florescence intensity image is reported. By means of the natural collagen auto-florescence it is possible to have a visualization and thus a statistic of the liposome filled compared of the total liposome produced without adding any external fluorescent probe that can prejudice the collagen matrix stability, the biocompatibility and can lead to toxicity.

After heating the sample at 37 °C (step five) to verify the collagen sol–gel transition and thus the

protein matrix formation inside the liposome cages we performed second harmonic generation microscopy. Indeed the collagen fibrils formation leads the molecules to assemble into large ordered noncentrosymmetric structures and thus a SHG signal rise after the fiber formation. In Fig. 2c a representative SHG image of collagen self-assembled spherical matrix is reported.

Because SHG does not involve excitation of molecules, it should not suffer, in principle, from phototoxicity effects or photobleaching, both of which limit the usefulness of fluorescence microscopy, including two-photon fluorescence microscopy (see emission spectra in Fig. 2d).

The size distribution can also be recovered and it is reported in Fig. 3b. Particles size ranges asymmetrically from 250 up to 2,500 nm.

To gain more statistical accuracy we also detect the particle size distribution from DLS measurements (Fig. 3a). Comparing the two distribution DLS is clearly more sensitive. To species in solution can be detected: liposomes with an hydrodynamic radius $R_h = (5 \pm 1)$ nm and collagen nanoparticles with an hydrodynamic radius $R_h = (340 \pm 100)$ nm where as, the SHG microscopy, can detect only the ordered noncentrosymmetric structures, thus the collagen nanoparticles.

When nanoparticles are finally produced, and the liposome removed by dialysis, the collagen matrix can be directly visualized by standard optical microscopy (Fig. 4a) or by atomic force microscopy to obtain single particles high resolution images (Fig. 4b) or structure details as the fibrils diameter or the matrix mesh size (Fig. 4c).
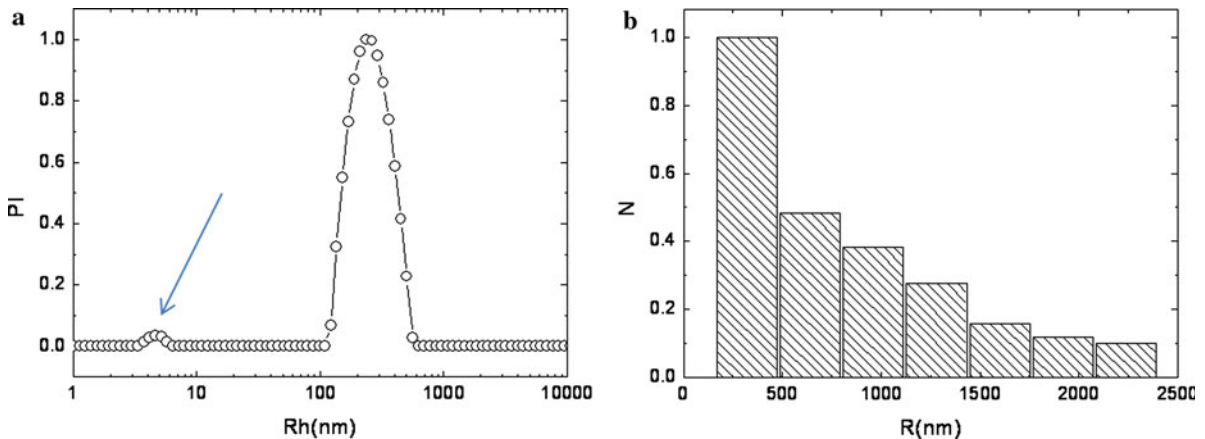

Fig. 3 Particles size distribution of collagen nanoparticles after the DMPC dissolution. In **a** the hydrodynamic radius distribution recovered by means of DLS is reported. Two different populations are observable: collagen nanoparticles at $R_h \sim 340$ nm and liposome at $R_h \sim 5$ nm (*arrow*). In **b** the radius distribution recovered by SHG confocal images is reported. Both the distributions are normalized and the frequency of the most probable value is set to one
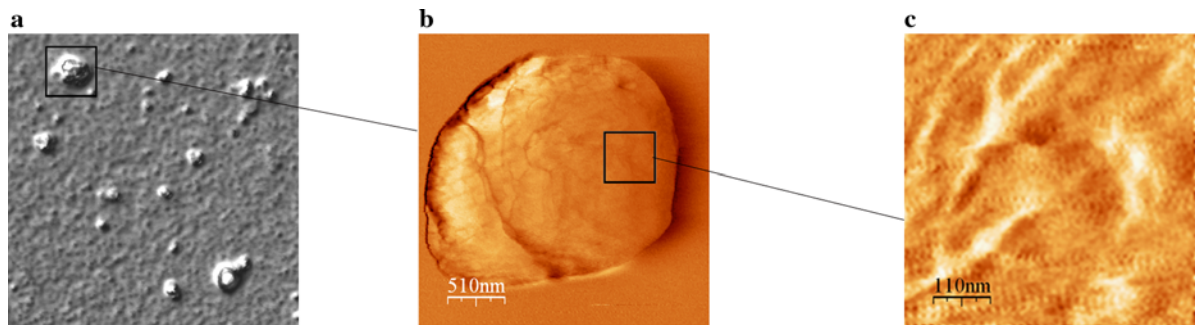

Fig. 4 Representative transmission intensity image (60 × 60 μm) (**a**) and representative atomic force microscopy deflection image of collagen aged particles 2 × 2 μm (**b**). Details of the polymers matrix forming the particles can be resolved (**c**)

Conclusion

For most of the industry's existence, pharmaceuticals have primarily consisted of simple, fast-acting chemical compounds that are dispensed orally (as solid pills and liquids) or as injectables. During the past three decades, however, formulations that control the rate and period of drug delivery (i.e., time-release medications) and target specific areas of the body for treatment have become increasingly common and complex. Because of researchers' ever-evolving understanding of the human body and the explosion of new and potential treatments resulting from discoveries of bioactive molecules and gene therapies, pharmaceutical research hangs on the precipice of yet another great advancement. However, this advance need, to be effective, the development of both new promising drugs and new mechanisms to deliver them (Vinogradov et al. 2006).

The current methods of drug delivery exhibit specific problems that scientists are attempting to address. For example, many drugs' potencies and therapeutic effects are limited or otherwise reduced because of the partial degradation that occurs before they reach a desired target in the body. Once ingested, time-release medications deliver treatment continuously, rather than providing relief of symptoms and protection from adverse events solely when necessary. Further, injectable medications could be made less expensively and administered more easily if they could simply be dosed orally. However, this improvement cannot happen until methods are developed to safely shepherd drugs through specific areas of the body, such as the stomach, where low pH can destroy a medication, or through an area where healthy bone and tissue might be adversely affected.

The goal of all sophisticated drug delivery systems, therefore, is to deploy medications intact to specifically targeted parts of the body through a medium that can control the therapy's administration by means of either a physiological or chemical trigger. To achieve this goal, researchers are turning to advances in the worlds of micro- and nanotechnology. During the past decade, polymeric microspheres, polymer micelles, and hydrogel-type materials have all been shown to be effective in enhancing drug targeting specificity, lowering systemic drug toxicity, improving treatment absorption rates, and providing protection for pharmaceuticals against biochemical degradation. In addition, several other experimental drug delivery systems show exciting signs of promise, including those composed of biodegradable polymers, dendrimers (so-called star polymers), electroactive polymers, and modified C-60 fullerenes (also known as "buckyballs").

Proteic compounds for drug delivery developed in recent years are specific, but also highly instable and sensible to external agents such as temperature, pH and UV radiations that can cause conformational changes or alterations of the active principle. Over the past few decades, there has been considerable interest in developing biodegradable nanoparticles as effective drug delivery devices. These nanoparticles are made of polymers able to form a porous matrix which controls release of drug. To allow substances reaching the target, nanoparticle can be administrated directly in situ or can be surface modified to bind specific receptors (Maham et al. 2009).

The production of these systems, however, often lead to instable matrix, or need complicated/drastic steps of preparation such us UV radiation or chemical treatments that render the compounds hardly manageable.

The collagen nanoparticles we developed can easily address many of the above issues. Indeed the production methods allows, in principle, the use of any protein for the gel matrix. This feature is crucial since it allows manage the gel mesh size in a wide range. Therefore, with respect to the size of the specific drugs to be released, we can always obtain the right mesh size for the desired delay in the drug's release.

In this context, it would be interesting that in case of a specific particles production (or functionalization), that require specific chemical agents or induce mechanical stress, laurdan can be a useful sensor able to monitoring the modifications that can occur, at the single particle level.

Furthermore, the possibility of shield the nanoparticle with a liposomal envelope open to the possibility of both protect from the outer environment drugs to be delivered and, by adding the appropriate tag, to a real site specific delivery.

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