

HYALURONATE NANOPARTICLES INCLUDED IN POLYMER FILMS FOR THE PROLONGED RELEASE OF VITAMIN E FOR THE MANAGEMENT OF SKIN WOUNDS

Gabriela Garrastazu Pereira¹, Cassia Britto Detoni¹, Anna Giulia Balducci², Valeria Rondelli³, Paolo Colombo³, Silvia Stanisçuaski Guterres¹, Fabio Sonvico^{2,3,4*}

¹ Faculty the Pharmacy, University Federal do Rio Grande do Sul, Porto Alegre, Brazil

² Biopharmanet-TEC, Interdepartmental Centre for Innovation in Health Products, University of Parma, Italy

³ Department of Pharmacy, University of Parma, Italy

³ Department of Medical Biotechnology and Translational Medicine, University of Milan, Italy

⁴ Graduate School of Health - Pharmacy, University of Technology Sydney, Australia

* Corresponding author

Graduate School of Health

Pharmacy

University of Technology Sydney

15, Broadway

Ultimo - NSW2007

Australia

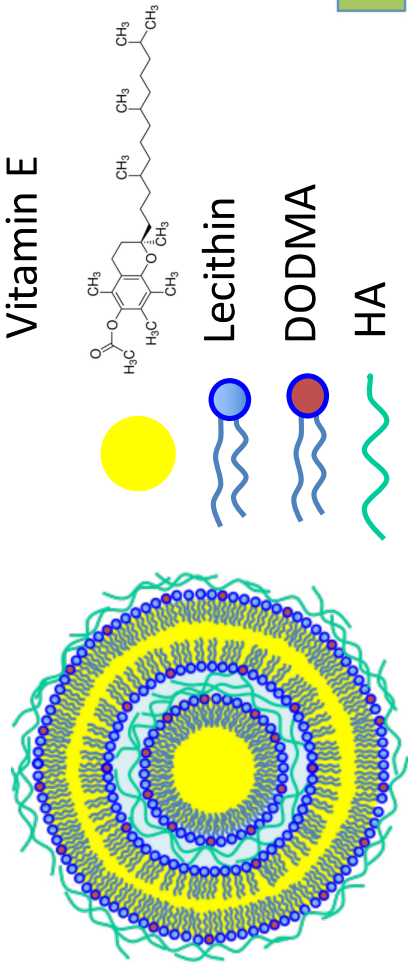
fabio.sonvico@uts.edu.au

Abstract

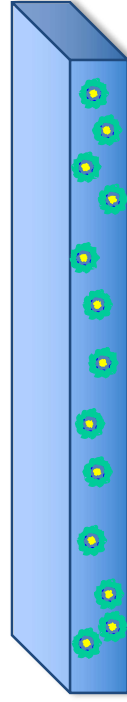
Lecithin and hyaluronic acid were used for the preparation of polysaccharide decorated nanoparticles loaded with vitamin E using the cationic lipid dioctadecyldimethylammonium bromide (DODMA). Nanoparticles showed mean particle size in the range 130 – 350 nm and narrow size distribution. Vitamin E encapsulation efficiency was higher than 99%. These nanoparticles were incorporated in polymeric films containing *Aloe vera* extract, hyaluronic acid, sodium alginate, polyethyleneoxide (PEO) and polyvinylalcohol (PVA) as an innovative treatment in skin wounds, such as burns. Films were thin, flexible, resistant and suitable for application on burn wounds. Additionally, in vitro occlusion study highlighted the dependence of the occlusive effect on the presence of nanoparticles. The results obtained show that the bioadhesive films containing vitamin E acetate and *Aloe vera* could be an innovative therapeutic system for the treatment of skin wounds, such as burns. The controlled release of the vitamin along with a reduction in water loss through damaged skin provided by the nanoparticle-loaded polymer film are considered important features for an improvement in wound healing and skin regeneration.

Keywords: Wound healing, Vitamin E, Hyaluronate nanoparticles, Polymer film, Local delivery, Burns

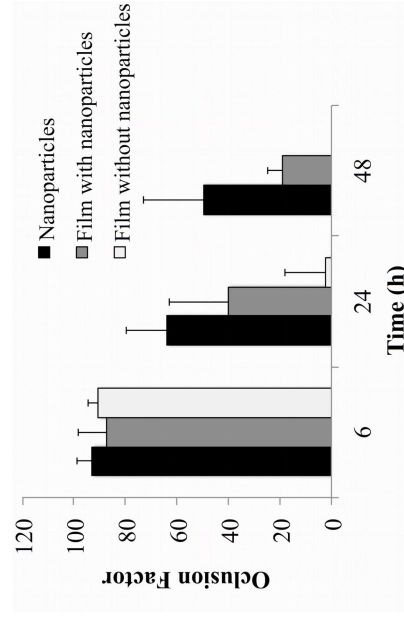
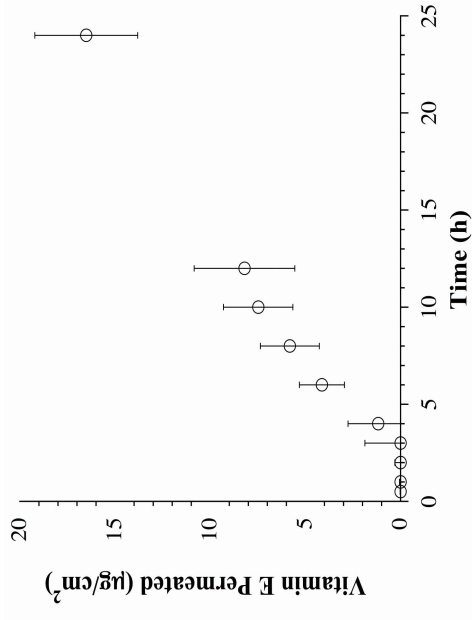
Hyaluronate nanoparticles can improve occlusion and control the delivery of vitamin E in bioadhesive polymeric films



Hyaluronate Nanoparticles



Polymeric bioadhesive film



INTRODUCTION

Delayed wound healing is one of the major therapeutic and economic issues in medicine today (Hanna & Giacobelli, 1997). Wound healing consists of a complex but very orderly array of overlapping phases in which highly specialized cells interact with an extracellular matrix to lay down a new framework for tissue growth and repair. There are four distinct phases of wound healing, which include hemostasis, inflammation, proliferation and remodeling (Hanna & Giacobelli, 1997; Andrew et al, 2004; Goldberg et al, 2010; Kiwanuka et al, 2012).

Natural products derived from plants and minerals remain a relevant source for obtaining medicines for the wound healing, like honey (Adewumi & Ogunjimi, 2011), propolis (Sforcin & Bankova, 2011), *Aloe vera* (Heck et al, 1981; Nadal & Bhardwaj, 2012; Feily & Namazi, 2009; Choi & Chung, 2003), hyaluronic acid (Kogan et al, 2007; Liao et al, 2005) and chitosan (Wood, 2012; Shi et al, 2006).

Aloe vera (*Aloe barbadensis* Miller) is widely used as a natural treatment and alternative therapy for various types of diseases, and several studies have suggested the healing, cosmetic, and nutritional benefits of this plant (Simal et al, 2000; Femenia et al, 1999; Hu et al, 2003, Eshun & He, 2004).

Vitamin E, a group of lipophilic substances including tocopherols and tocotrienols, has elicited a significant interest for its role in burn injuries, in particular for its antioxidant action during the tissue reperfusion that contribute to exacerbate the injury initiated by ischemia. In fact, reactive oxygen species and free radicals are produced during the phase of reperfusion of ischemic tissues, damaging numerous cell components, including nucleic acids, lipids and proteins (Berger, 1983). The last step of a burn treatment is generally the application of a bandage. Ideally, the dressing should fill the wound, provide a moist environment, absorb excess exudate, provide thermal insulation and a barrier to bacteria and be minimally traumatic for application and removal (Peter, 2001; Wolf et al, 2011).

We recently developed a polymer film based on sodium alginate and polyvinyl alcohol (PVA) loaded with *Aloe vera* and vitamin E, able to release the 30% of the vitamin in 12 hours and to accumulate more vitamin in the deep layer of the skin at 2 and 4 hours when compared to a conventional cream. However, the film presents a burst release of the vitamin and is not occlusive (Garrastazu et al., 2014).

The combination of PVA with sodium alginate provides to the films proposed the ability to entrap water, the transparency and adequate mechanical characteristics to

the dry film, along with the possibility of forming a fibrous gel when in contact with wound exudate or blood (Xue et al., 2013; Dong et al, 2006; Mandelbaum & Mandelbaum, 2003). Another component of the proposed burn dressing film is sodium hyaluronate, which promotes the mobilization of cells modulating the inflammatory response and stimulating angiogenesis (Kogan et al, 2007; Liao et al, 2005). In order to maintain the polymer composition, structure and biopharmaceutical properties of the film, in order to control the release of the antioxidant tocoferol, it is proposed to encapsulate the vitamin E acetate in novel hyaluronate nanoparticles.

Innovative and carefully conceived nanoparticles could lead to a highly dispersed form of vitamin E, characterized by high specific surface and providing intimate contact with the wounded tissue and cells involved in the healing process.

The incorporation of nanoparticulate drug delivery systems in polymer films containing PVA, alginate and hyaluronate in view of a topical application on burns, combines the advantages of the three polymers in a device for an extended release of drug.

The aim of the present work was to develop and characterize vitamin E loaded-nanoparticles that could be subsequently embedded in a bioadhesive polymeric film containing *Aloe vera* extracts. In particular, nanoparticles based on hyaluronate and lecithin were prepared by direct injection of vitamin E and soybean lecithin alcoholic solution with different amount of dioctadecyldimethylammonium bromide (DODMA) into hyaluronic acid water solution. The nanoparticles were obtained from the supra-molecular self-organizing interaction of the positively lipid material in presence of the negatively charged polysaccharide and studied for their physico-chemical properties, stability and encapsulation efficiency, inclusion in the polymeric film and release of the vitamin.

MATERIALS AND METHODS

Materials

Aloe vera spray dried powder 200:1 *Aloe*:mannitol was obtained from Brasquim (Porto Alegre, Brazil). Vitamin E acetate (alpha tocopherol acetate) was purchased from ACEF (Fiorenzuola, Italy), hyaluronic acid (High MW, 6×10^6 Da) solution 1% w/v, density $0.900 - 1.100 \text{ g/cm}^3$ was obtained from DEG (São Paulo, Brazil); soybean lecithin (Lipoid S45) was obtained from Lipoid AG (Ludwigshafen, Germany); dioctadecyldimethylammonium bromide (DODMA) (>98%) was supplied by Sigma-Aldrich (St. Louis, USA); polyvinyl alcohol (PVA) of molecular weight of 83,400 Da from Nippon Gohsei (Osaka, Japan), sorbitol solution 70% from ACEF (Fiorenzuola, Italy), alginic acid (Satalgine[®]) from Cargill (Saint Germain-en-Laye, France) and poly(ethylene oxide) water soluble resin (PEO 12 NF[®]) from Union Carbide (Milan, Italy). MilliQ ultrapure water (Millipore, Billerica, USA) was used for all experiments. All other chemicals are of analytical grade.

Nanoparticle preparation

Nanoparticles were obtained with an injection method described previously (Senyigit et al, 2010). Nanoparticle suspensions were obtained by injecting through a glass pipette (internal diameter 0.75 mm, injection rate $40 \text{ ml} \cdot \text{min}^{-1}$, syringe pump model 200 KD Scientific, Holliston, USA) 8 ml of a lecithin ethanol solution ($25 \text{ mg} \cdot \text{ml}^{-1}$) containing different concentration of DODMA (0.025; 0.05; 0.1; 0.25; 0.5; 1 and 2% w/v) and $2.0 \text{ mg} \cdot \text{ml}^{-1}$ vitamin E into 92 ml of a hyaluronic acid solution (1% w/v) stirred with a high-performance dispersing instrument operated at 15,000 rpm (Ultraturrax TP 18/10–10N, IKA Werke, Staufen, Germany).

Nanoparticle morphology

Nanoparticles were analyzed with a transmission electron microscope (EM 208S, Philips, Eindhoven, The Netherlands) operated at 80 kV. The colloidal suspension was diluted tenfold with distilled water; an aliquot of $15 \mu\text{l}$ was then deposited on a Formvar coated grid (300 mesh, AGAR Scientific, Stansted, UK) and stained with a phosphotungstic acid solution 2% (w/v) for 20 seconds. The excess of reagents was delicately removed by means of filter paper.

Physico-chemical characterization of nanoparticles suspensions

Vitamin E-loaded nanoparticles were characterized by measurement of particle size, size distribution (polydispersity index - PDI) and zeta potential. Measurements of particle size (photon correlation spectroscopy, PCS) and zeta potential (Phase Analysis Light Scattering, PALS) were performed at 25 °C using a Zetasizer Nano ZS (Malvern Instruments, UK) at an angle of 173°. The particle size, size distribution and polydispersity index were determined in diluted samples (500 times) with filtered ultrapure water (0.45 µm cellulose acetate filters). The zeta potential measurements were performed after diluting the samples (500 times) with 10 mM NaCl aqueous solution and setting the measurement time at 360 seconds.

Laser diffractometry of nanoparticles suspension

The particle size was evaluated also by laser diffractometry (LD) method (Mastersizer 2000, Malvern Instruments, Malvern, UK) to detect the possible presence of microparticles. The volume weighted mean diameter ($D_{4,3}$) was obtained. The size distribution was evaluated by calculating the span, using Equation (1).

$$span = \frac{d_{90} - d_{10}}{d_{50}} \quad \text{Equation 1}$$

where d_{90} , d_{10} and d_{50} are the 90%, 10% and 50% cumulative volume distribution respectively. Thus, the span gives a measure of the range of the volume distribution relative to the median diameter. All samples were analyzed in triplicate batches ($n=3$).

Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) is a method allowing the visualization and analysis of nanoparticles in liquid dispersions (NanoSight LM10 equipped with NTA 2.0 Analytical Software, NanoSight Ltd., Salisbury, UK). This technique measures particles in the range of 10–2000 nm. NTA is carried out using a small amount of the diluted samples (0.5 ml) introduced into the chamber by a syringe. The chamber is placed on an optical microscope and the particles are illuminated by a laser beam (635 nm wavelength). The video images of the Brownian motion of the individual particles are followed in real-time via CCD camera and analyzed using the NTA 2.0 Analytical Software (NanoSight®). The digital camera captures the light scattered by

the particles and the motion of each particle is tracked from frame to frame. The rate of particle movement is related to a sphere equivalent hydrodynamic diameter calculated using a variation of Stokes–Einstein equation taking into account the two dimensional particle tracking (see Equation 2).

$$\overline{(x, y)^2} = \frac{4TK_b t}{3\pi\eta d} \quad \text{Equation 2}$$

In Equation 2, the mean squared particle displacement of a particle over time (t) is related to its hydrodynamic diameter (d), being T and η the sample temperature and viscosity respectively. The particle size distributions are calculated on a particle-by-particle basis. Visible particles in the frame correspond to each separate light scattering center that is seen as an individual particle during filming (Filipe et al, 2010).

The formulations were diluted 5000 times using ultrapure water and each video clip was captured over 60 seconds. The automatic detection threshold was enabled and the maximum particle jump was set at 10 in the software. All measurements were performed in triplicate. The polydispersity of the particle sizes was calculated as Span, which is the width of the distribution based on the 10%, 50% and 90% of the cumulative size distribution, as presented in Equation 1.

Determination of encapsulation efficiency

The encapsulation efficiency of vitamin E into nanoparticles was determined indirectly by ultrafiltration method using centrifugal filter tubes (Amicon Ultrafree CL, 10,000 MW, Millipore, Japan). The percentage of encapsulated substance was calculated by the difference between the total amount of vitamin E in the formulation, which was measured after the dissolution of the nanoparticles with methanol, and the free concentration of vitamin E that remained in the filtrate aqueous phase after ultrafiltration (Vivaspin cut-off 10,000 Da, Sartorius SpA, Bagno di Ripoli, Italy) by centrifugation (5 minutes 5000 rpm, Centrifuge 5417R, Eppendorf, Sao Paulo, Brazil) of the nanoparticles, divided by the total amount of vitamin E in the nanoparticles multiplied by 100. Analysis of vitamin E was performed by the validated HPLC method reported below.

Evaluation of suspension stability by multiple light scattering

To check the colloidal physical stability, the nanoparticle suspensions were evaluated by multiple light scattering using a Turbiscan Lab (Formulaction, France). In this method the light source is an electro luminescent diode in the near infrared (880 nm wavelength) and two synchronous optical sensors receive, respectively, the light transmitted through the sample (0° from the incident light, transmission sensor), and the light backscattered by the sample (135° from the incident radiation, backscattering detector), acquiring transmission and backscattering data every 40 µm from the bottom to the top of the cell (Mengual et al, 1999). The samples (10 ml) were poured into the glass cells without any treatment or dilution and analyzed at 25° C for 1 h using the scan mode.

Stability Studies

The suspensions of nanoparticles containing Vitamin E and prepared with concentrations of DODMA in the range 0.025 - 0.25% w/v were evaluated for their stability at room temperature and protected from light. The average particle diameter and polydispersity index of these suspensions stored at room temperature and sheltered from light, were determined at 1, 30, 60, 90 days. All suspensions were analyzed in triplicate.

Polymer film preparation

Polymer films were prepared as reported previously with slight modifications (Garrastazu et al., 2014). The composition of the dried films is reported in **Table 1**. The films were prepared starting from two solutions:

- Solution A: alginate powder was added to 20 ml of 1% solution of hyaluronate and stirred until complete dissolution. *Aloe vera* was then added and homogeneously dispersed.
- Solution B: PEO, PVA and sorbitol were added to 10 ml of Vitamin E-loaded nanoparticles suspension under magnetic stirring and gentle heating until complete solubilization. The polymer solution obtained was allowed to stand for 4 h, until all trapped air bubbles were removed.

Solutions A and B, prepared as described were mixed under magnetic stirring for 4 hours. The polymeric films were then produced by layering the obtained viscous

solution using a variable opening casting knife (gap 2 mm, BYK Gardner, Lainate, Italy) on a polyester translucent polyethylene laminate film (Scotchpak 1220 Backing, 3M Italia, Italy) and by subsequent drying for 8 h in an oven (55°C).

In order to analyze the influence of the embedded nanoparticles on films occlusion properties, films were produced with and without vitamin E-loaded nanoparticles for the *in vitro* occlusion test.

[Insert Table 1 here]

Physical characterization of the film

At least 3 disks of material (15 mm of diameter) were sampled by die cutting the polymeric film produced. Each disk was accurately weighed and its thickness measured (Absolute Digimatic 547-401, Mitutoyo, Milan, Italy, sensitivity 0.001 mm). Residual water content of each formulation was determined using Karl-Fisher titration (TitroMatic KF 1S, Crison, Spain).

Physical characterization of the nanoparticles released from the film

At least 3 disks of the nanoparticles containing film (15 mm of diameter) were sampled. The samples were then dissolved in 10 ml of water under mild magnetically stirring. The samples were analyzed by PCS (Zetasizer Nano ZS, Malvern Instruments, UK) as reported before in the text.

Vitamin E acetate assay

For each film formulation at least 3 disks (15 mm of diameter, surface area 176.7 mm²) were sampled. Every disk was accurately weighed and the sample then dissolved in 10 ml of water: ethanol mixture (20:80 v/v) under sonication for 2 h. The solution obtained was analyzed by HPLC in order to determine the amount of vitamin E acetate contained in the film. Vitamin E acetate analysis was performed by HPLC according to a method previously described.

Briefly, vitamin E acetate analysis was performed by HPLC using the following experimental conditions: column LiChrospher® 100 RP18: 5 μm, 250 mm × 3 mm (Merck, Germany), mobile phase: methanol:isopropanol (50 : 50 v/v), flow rate of 0.7 mL/min, and UV detection at 285 nm. Samples were filtered through 0.45 μm (Ultracel regenerated cellulose, Microcon Filters, Merck Millipore, Darmstadt,

Germany) and were injected (20 μL) in an Agilent 1200 Series HPLC System (Agilent Technologies Italia, Cernusco sul Naviglio, Italy). In these conditions, the retention time was about 6.6 min. System suitability was checked according to the USP 24. The detector response was linear from 3 to 51 $\mu\text{g}/\text{mL}$, the limit quantification of 0.42 $\mu\text{g}/\text{mL}$, a limit of detection of 0.13 $\mu\text{g}/\text{mL}$ and r^2 0,9995 (Garrastazu et al, 2014).

The loading of vitamin E acetate in polymeric films was expressed as percentage by weight (% w/w).

Scanning Electronic Microscopy Analyses

Film samples were put on a sample holder and examined with a JEOL JSM 6400 (Tokyo, Japan) scanning electron microscope (SEM) at an intensity of 15kV using a range of magnifications from 200x to 10000x.

Atomic Force Microscopy Analyses

The films surface morphology was studied by AFM. For AFM observations, films were placed on a magnetic sample holder using adhesive tape. Films were examined using XE-100 AFM (Park Systems, Suwon, South Korea). The sample holder containing the sample was put in the XY scanner of the AFM and a CCD camera was used to localize film surface. Images were acquired in air with non-contact mode. The spring constant was typically $0.08 \text{ N}\cdot\text{m}^{-1}$ and samples were scanned at constant force with a low scan rate (0.76 Hz) in order to reduce noise and minimize sample damages. Images were obtained with 256x256 pixels of resolution and image processing (line-wise flatten only) was performed in XEP – Data (Park System, Suwon, South Korea). Height images were displayed as 3D-views and the surface scanned was $5 \times 5 \mu\text{m}$.

***In vitro* occlusion test**

The *in vitro* occlusion test used was modified from the one proposed by Wissing and Müller (2002). Briefly, a 20 ml flask was filled with 10 ml water and sealed with a cellulose acetate filter (J Prolab, José Dos Pinhais, Brasil, cut-off size 3 μm). Film formulations containing vitamin E nanoparticles and without the nanoparticulate formulation were applied on the filter and stored at 32 °C and 50% relative humidity for 48 h. At predetermined time points the flask is weighed and the water loss

determined. A flask without formulation is used as control. The occlusion factor F was calculated according to Equation (3) after 6, 24 and 48 h.

$$F = \frac{A-B}{A} * 100 \quad \text{Equation 4}$$

Where A is the water loss without formulation and B is the water loss in presence of the film. Every experiment was carried out in triplicate.

***In vitro* vitamin E release studies**

A release assay of total vitamin E (drug encapsulated in nanoparticles and free drug in solution), the method did not allow to differentiate between the two forms) from films was performed using modified Franz permeation cells with a synthetic membrane of regenerated cellulose with 0.45 µm pores (Merck Millipore, Darmstadt, Germany) to separate the donor and acceptor compartment. The *in vitro* release studies were conducted on vertical diffusion Franz cells, having a receptor compartment with a capacity of about 12.0 ml and a diffusion area of 2.3 cm² (Xue et al, 2013; Dong et al, 2006). The cellulose membrane was kept in contact with a receptor solution phosphate buffer pH 7.2 with 10% ethanol and 0.5% polysorbate 80 at a temperature of 37°C (± 1°C) and constantly stirred with a magnetic bar at 100 rpm. The acceptor medium was selected by determining the saturation concentration of vitamin E acetate in different candidate receptor solutions, selecting the one that provided the highest solubility, i.e 24.4 mg/ml.

For this study films were used containing approximately 320 µg vitamin E acetate (approximately 2.0 cm² diameter disks). The material under investigation was placed in the donor compartment and hydrated with 500 µl of phosphate buffer pH 7.2. The film dipped in the buffer hydration from both surfaces and the formation of a gel in contact with the membrane.

At pre-established time intervals (each hour up to 12 hours), 1 ml of the medium was removed and replaced by an equal volume of preheated fresh receptor solution. Samples were filtered through 0.45 µm filters (RC, Merck Millipore, Darmstadt, Germany) before the determination by HPLC of their total vitamin E acetate content according to the method described before. The results report the average of five experiments.

RESULTS AND DISCUSSION

In this work the formation of lecithin/hyaluronate nanoparticles does occur because of an electrostatic interaction between the components. The production method used is similar to the experiments performed by Sonvico and colleagues (Senyigit et al., 2010; Gerelli et al., 2008), that exploited the interaction between a negative phospholipid and a positive polysaccharide to form lecithin/chitosan nanoparticles. The particles formed were multilayered and characterized by alternating phospholipids lamellae and chitosan aqueous layers (Gerelli et al., 2008b).

In the formulations proposed small amounts of a positively charged amphiphilic molecule, DODMA, generally used to produce cationic vesicles (Zhang et al, 2004), has been used to assure the interaction between the phospholipid phase with the negatively charged hyaluronate.

Nanoparticles prepared with various concentrations of DODMA showed different characteristics in terms of size and surface charge. In the photomicrographs obtained from the vitamin E loaded nanoparticles suspension, the presence of spherical structures with particle diameter around 220 nm (Figure 1) were observed in batches containing 0.05 to 0.25% DODMA. On the contrary, the formulations containing more than 0.5% DODMA did not show the formation of individual nanometric structures, but larger agglomerates and clusters.

[Insert Figure 1 here]

Figure 2 shows the mean particle size and zeta potential of nanoparticles determined by PCS. The diameters obtained by PCS were slightly larger than those observed by electron microscopy, since this technique determines the hydrodynamic radius of the particle while the electron microscopy is performed on a dried sample (Colomé et al., 2011). Particles with 1.0 and 2.0% of DODMA presented a particle size slightly higher than 200 nm and positive charge. However, these formulations evidenced the tendency to agglomerate after few days of storage.

The particles containing 0.5% of DODMA present large positively charged aggregates with highly variable particle size, that make impossible the measure with PCS.

On the contrary, the particles produced with 0.025, 0.05, 0.1 and 0.25% DODMA presented small and narrow particle size between 100 and 200 nm, negative surface charge and stable suspensions. For all formulations, PDI values varied from 0.12 to 0.20 indicating relatively narrow size distributions.

[Insert Figure 2 here]

Nanoparticles formed by DODMA, lecithin and hyaluronate are structures formed by the attractive interactions between the polar heads of the lipid amphiphilic molecules and charged polysaccharide while the hydrophobic portions of lipids are interacting with the lipophilic vitamin E, giving rise to a core shell particle (Gerelli et al, 2008). This is evidenced by the data presented in Figure 2, for lower quantities of DODMA the nanoparticle charge is negative because the hyaluronate is providing enough negative charges to neutralize the positive charge of the lipophilic positively charged excipient DODMA. However, a clear reduction of the negative charge with the increase of DODMA in the formulation is evidenced. For preparations containing 0.5% of DODMA or more, the total net charge of the particles becomes positive. In these formulations, the positive charges provided by the quaternary ammonium compound DODMA are in excess, but apparently they are not able to provide long-term stabilization to the structures formed (Figure 2).

Hyaluronate-based nanocarriers for the delivery of macromolecules and genes have been developed by the group of Professor M.J. Alonso at University of Santiago de Compostela, Spain (de la Fuente et al, 2008; de la Fuente et al, 2008b). The same group developed recently a nanocarrier system based on self-assembly of components by electrostatic interaction for the intracellular delivery of anticancer drugs. The nanocarriers obtained were defined hyaluronan nanocapsules and described as having a polymer shell around an oily core (Oyarzun-Ampuero et al., 2013). Concerning the structure of the nanocarriers obtained here using vitamin E, lecithin, DODMA and hyaluronate it could be assumed, at first glance, that a similar structure was obtained.

However, in previous studies with chitosan nanoparticles, it was shown that an alternative structure is possible in which multilayer disposition of lipids and polysaccharide is obtained (Gerelli et al., 2008b).

Some recent data obtained at the European Synchrotron Research Facility in the case of a formulation similar to the one presented (hyaluronate, DODMA, lecithin and an oily phase containing medium chain triglycerides and glycerol monolinoleate) suggest that this structure is present also in the case of hyaluronate nanoparticles with or without an oily component (see Supplementary Data). The supposed structure for the vitamin loaded nanoparticles is not then a nanocapsule structure, but a multilayer particle alternating layers of lipids and hydrated hyaluronate.

[Insert Table 2 here]

Laser diffraction was carried out to assess the simultaneous presence of microparticles in the nanoparticles formulations (**Table 2**), since PCS analysis is limited to particle size ranging from 3 to 3000 nm and sometimes overlook bigger agglomerates (Poletto et al, 2009). The volume distribution diameter for 90% of particles did not exceed 500 nm for the formulations up to 0.25% DODMA and no micrometric particles were detected for these formulations. Span values indicated narrow size distribution of the colloidal particles formed. Additionally, $D_{4,3}$ values, which represent the volume moment mean diameter of the distribution, were similar to mean diameter values determined by PCS for particles prepared with up to 0.1% DODMA. The particle size distributions by volume showed monomodal size distribution for vitamin E loaded nanoparticle suspensions. The results indicated that for these formulations no micron-sized particles were formed.

On the contrary, nanoparticles produced with 1 and 2% DODMA presented much higher span values and larger $D_{4,3}$ diameters suggesting the presence of agglomerates. At the same time the preparation containing 0.25% DODMA, despite providing a relatively low span and d_{10} , d_{50} and d_{90} values, showed a $D_{4,3}$ slightly above the micron probably as a consequence of the reduced surface charge, just below -20 mV. The zeta potential, the electric potential at the plane of shear, can predict the physical storage stability of colloidal systems. According to previous studies, the colloids having zeta potential values higher than -30 mV show good physical stability during the shelf life, being optimal when they reach approximately -60 mV (Teeranachaideekul et al, 2008; Chen et al, 2006). In this study, the three nanoparticles formulations prepared using 0.025, 0.05 and 0.1 % DODMA present good values of zeta potential (**Figure 2**), which were around -40 mV, indicating that

these formulations show better physical stability since the electrostatic repulsion between the particles prevents the particle aggregation. It is noteworthy that the measurements were carried out by direct dilution of each sample into the wet sample dispersion unit of the instrument without previous filtration or centrifugation.

[Insert Figure 3 here]

As a consequence, vitamin E-loaded nanoparticles prepared with up to 0.1% DODMA were considered suitable formulations for follow-up studies, since they are composed of self-assembled biocompatible materials presenting particle sizes lower than 200 nm. These characteristics could play a pivotal role in the efficacy of a formulation for topical administration (Angelini et al, 2011; Jones, 2005). The importance of the size of nanoparticles relies on the greater the surface area of contact provided by smaller particles, which in the case of wounds could be critical for the healing process.

The size distribution of vitamin E loaded nanoparticles suspension was also investigated by NTA method for nanoparticles prepared using 0.05 and 0.1% DODMA. A microscopic view of the vitamin E loaded nanoparticles suspension illuminated by the laser diode is shown in **Figure 3**. The scattered light in the microscope field of view related to vitamin E loaded nanoparticles suspension Brownian motion are very similar, indicating particle size in a same range, which was confirmed by the distribution data. Besides, the mean size values obtained by NTA, well dispersed and spatially resolved particles were visualized in the frame images obtained from the random motion of the particles in suspension captured using a CCD camera for 0.05% DODMA (temperature 25.1°C and viscosity 0.89 cP) and 0.1% DODMA (temperature 24.4°C and viscosity 0.90 cP). These images show a microscopic view of the scattering particles illuminated by the laser diode. Larger particles scatter more light and larger luminous dots appeared in the video frame, while smaller particles move faster and longer distances with respect to larger ones (Jakubowicz, 2008; Gallego-Urrea et al, 2010). Even though different intensities of light scattering can be observed for each formulation, cumulative data obtained for the percentile of the particle distribution by NTA showed diameters in a narrow size distribution. The different dilution ratios used for the DLS and NTA analyses did not

influence the average particle size results. Comparing DLS and NTA, similar sizes of particles were determined.

[Insert Table 3 here]

Vitamin E content in the nanoparticles preparations was nearly 2.0 mg.ml⁻¹ (**Table 3**). Encapsulation efficiency values were close to 100% for all vitamin E loaded nanoparticles. In general, lipophilic drugs, like vitamin E, dissolve in the lipids and have often been selected for incorporation into lipid nanoparticles in order to have high drug loading and entrapment efficiency (Esposito et al, 2008). However, this does not necessarily guarantee that high encapsulation efficiencies are maintained, because some authors observed drug expulsion and precipitation or phase separation (Almeida and Souto, 2007). As a consequent the stability of vitamin E loaded nanoparticles has been assessed.

Multiple light scattering analysis by Turbiscan[®] can be used to determine reversible (creaming and sedimentation) and irreversible (coalescence and precipitation) destabilization phenomena directly in the formulation without any prior dilution or treatment more easily than other techniques. Vitamin E loaded nanoparticles suspension containing 0.05 and 0.1% DODMA are milky and no transmission was detected by multiple light scattering analyses. Thus, variations in backscattering were used to evaluate suspension stability. Negative variations in the relative backscattering at the top of the cells were observed. However, both nanoparticles suspensions showed backscattering variations lower than 5%, indicating stable formulations (Celia, 2009). The small values variations, determined during the experimental period, indicated slow sedimentation or creaming. No variation at the middle of the cell was observed indicating the absence of flocculation, coalescence or phase separation.

In addition to Turbiscan measurements, stability studies at room temperature for up to 90 days were conducted in order to determine the behavior of 0.025, 0.05, 0.1, 0.25% of DODMA systems in terms of size, surface charge and polydispersity index. The average diameter of particles presented to around 200 nm and no increase with storage time was evidenced, with the only exception of nanoparticles containing 0.25% DODMA that presented a slight increase of size up to around 300 nm since the first month, but did not show any further size increase or agglomeration (**Figure**

4a). As observed for the mean particle diameters, polydispersity indexes did not vary significantly during storage time, remaining between 0.15 and 0.25 (**Figure 4b**), thus indicating that no significant aggregation of nanoparticles and widening of particle size distribution occurred. Zeta potential was monitored as well up to 30 days without evidencing any significant variation (data not shown). These results indicate that the studied formulations show no tendency to aggregation or flocculation in the range of amounts of DODMA employed.

[Insert Figure 4 here]

The formulation of nanoparticles containing 0.1% of DODMA was chosen for inclusion in films and further studies, because these nanoparticles present suitable characteristics for a topical use. Nanometric systems have high surface area and are considered vectors for the administration of lipophilic substances and enabling a homogeneous release, thus increasing the therapeutic response at the site of action for a prolonged time.

Film Characterization

An accurate determination of the thickness of the film is important because it serves as a basis for calculation of several functional properties of the films and is critical for the analysis of manufacturing repeatability. Thickness, weight and residual water content of the polymeric film are shown in **Table 4**. The films were thin, presenting thickness lower than 150 μm and had a weight per square centimeter close to 80 $\text{mg}\cdot\text{cm}^{-2}$. The amount of vitamin E acetate in each film was about $2.00 \pm 0.05\%$ by weight.

They were flexible and resistant and could be easily handled, cut in the desired shape and eventually deformed to adapt to the application site.

[Insert Table 4 here]

Figure 6 shows the details of the surface of nanoparticle-containing film, investigated using both atomic force and scanning electron microscopy. The films present a rough surface and are characterized by more pits and depressions and of smaller dimensions. The micrometric roughness of the surface of the films could possibly

allow them to adhere to the healing tissue, which leads to a larger contact surface and eventually increases the time that the films stay in contact with the lesion, therefore enabling the promotion of a more efficient cicatrix of the burn wound (Figure 6b).

[Insert Figure 6 here]

Figure 7 shows the *in vitro* calculated occlusion factor due to the use of a polymeric film containing Vitamin E-loaded nanoparticle and without nanoparticles. Significantly higher ($p < 0.05$) occlusion factor values (40.0 ± 22.0 vs. 2.1 ± 15.9) were measured when the nanoparticles were incorporated in polymeric films compared to polymeric films alone after 24 h. Furthermore, 48 h after film application was observed that only the polymeric films with vitamin E-loaded nanoparticles produced a detectable occlusion. This is in accordance to previous studies, where it was found that lipid nanoparticles not only could provide occlusion, but the occlusion factors values obtained were inversely proportional to their particle size, i.e. occlusion increased with the decrease of their average particle diameter (Wissing and Müller, 2002). One of the problems that occur in burn wounds is the increase in trans-epidermal water loss as a consequence an occlusive dressing appears beneficial. When lecithin/hyaluronate nanoparticles are used as carriers for vitamin E topical administration, the occlusive effect due to film application on the lesion surface will help to reduce the trans-epidermal water loss (Bhaskar et al, 2009).

[Insert Figure 7 here]

Regarding the *in vitro* release studies, the diffusion of vitamin E from polymeric films containing nanoparticles through a model hydrophilic membrane showed a sustained profile ($16.5 \pm 0.32 \mu\text{g}/\text{cm}^2$ membrane after 24h) (**Figure 8**). The release rate of vitamin E nanoparticles from polymeric films was significantly lower than the diffusion of the free drug in similar polymeric films reported previously ($54.00 \pm 0.60 \mu\text{g}/\text{cm}^2$ membrane after 12h). It was also shown by complete dissolution of the nanoparticles loaded polymeric films in excess water that vitamin E was released in form of particles with a diameter and size distribution (around 150 nm with polydispersity index lower than 0.15) very similar to the initial nanocapsules, demonstrating the

absence of particle agglomeration in the film matrix. In comparison, the formulation prepared in the previous paper (Garrastazu et al., 2014) proposed the direct dispersion of vitamin E in the film components. The vitamin was dispersed in the polymer solution without controlling droplets size and leading most likely to a traditional micron-sized emulsion. This led to a relatively poor control of the release of the vitamin E.

[Insert Figure 8]

The polymeric films containing nanoparticles are not common, but it is possible to see with the results that the nanoparticles sustained more the release of the drug compared with a free drug. Because of the lipophilic characteristic, vitamin E presents high affinity to the phospholipid matrix of the nanoparticles produced. Likewise, another study (Bhaskar et al, 2009) evaluated the release of a highly lipophilic drug, amphotericin B, in dialysis bag and found out slow release rate, about 60% in 25 days.

Besides nanoparticle, polymeric films could also contribute to slow the release of vitamin E, which allows the dressings to remain longer in place and to be replaced less frequently. Polymeric films are also known like systems that sustained drug release. According to Lei et al (2010) polymeric films presented good performance of a multilayered structure for drug controlled releasing stent application was demonstrated by investigating the release and permeation behaviors of a series of multilayered 5-FU-loaded poly(ϵ -caprolactone) films.

Agarwal et al (2012) produced some films containing chlorhexidine for the treatment of wound and the films presented a good release that permitted to treat the wound better than the free chlorhexidine.

The particles and the films, when relying on the skin may suffer the actions on enzymes present in wounds so as exudate present in the healing process and can thus releasing vitamin E more quickly. In any case, the nanoparticles proposed allow sustained release of vitamin E at the site of the lesion, which can be useful for the regeneration process in burns healing. In fact, in burns the oxidative stress is a factor perpetuating the inflammatory response and may cause a gradual worsening of the injury. In addition, the extract of *Aloe vera* loaded in the film contains proteins,

carbohydrates such as mucopolysaccharides, vitamins and minerals. These nutrients will act synergistically with the other components of the formulation, i.e. vitamin E nanoparticles, alginate and hyaluronate for the hydration, regeneration and re-epithelialization of the damaged skin. Specifically, these features appear to be very important in the treatment of burn wounds, where the damage is often more extended and attains deeper layers of the tissue.

Thus, any substance, be it a natural product, synthetic compound or drug that demonstrates stimulate antioxidant defenses and decrease the production of free radicals, constitutes an important object of study for the treatment or prevention of disease. In this case vitamin E was used in order to prevent oxidation of the membranes, accelerate healing, affect the production of collagen fibers and prevent the formation of hypertrophic scars.

Conclusion

A new type of hyaluronic acid coated lipid nanoparticles has been developed for the topical administration of vitamin E. Nanoparticles containing 0.1 % of the cationic lipids DODMA showed size below 200 nm, narrow size distribution and stability for up to three months. Those nanoparticles guarantee a slow release of the vitamin and could be embedded efficiently in a polysaccharide film formulation for topical administration. The slow release showed by nanoparticles was maintained in the film formulation and the system showed also more efficient occlusion properties compared to a formulation containing the vitamin but no nanoparticles.

In addition, nanoparticles embedded in the formulation could further protect the vitamin against degradation from light and oxygen and provide a slow and progressive delivery of the active towards regenerating skin layers.

The formulation developed seems ideal to provide a progressive and efficient release of wound healing substances in a localized and optimal delivery to the regenerating cellular skin layers.

Figure Captions

Fig. 1 TEM images of nanoparticles suspension (A) 0.05% DODMA vitamin E-loaded nanoparticles, (B) 0.1% DODMA vitamin E-loaded nanoparticles (C) 0.5% DODMA vitamin E-loaded nanoparticles.

Fig. 2 Mean particle size (nm) and zeta potential values determined by PCS of vitamin E-loaded nanoparticles suspensions.

Fig. 3 Particle size distribution (nm) (left) and sample video frame (right) obtained from NTA for 0.05% DODMA vitamin E-loaded nanoparticles (A) and 0.1% DODMA vitamin E-loaded nanoparticles (B).

Fig. 4 Mean nanoparticles diameter (A) and polydispersity index (B) of nanoparticles containing increasing concentrations of DODMA after 1, 30, 60 and 90 days of storage at room temperature (n = 3).

Fig. 6 Polymeric films with vitamin E loaded nanoparticles: (A) SEM image and (B) AFM image of polymeric film surface.

Fig. 7 Occlusion factor of polymeric film with (■) and without vitamin E-loaded nanoparticles (□) after 6, 24 and 48 h.

Fig 8. *In vitro* permeation study of vitamin E-loaded nanoparticles from the polymeric films through through a membrane of regenerated cellulose with 0.45 μm pores (up to 24h).

Figure 1

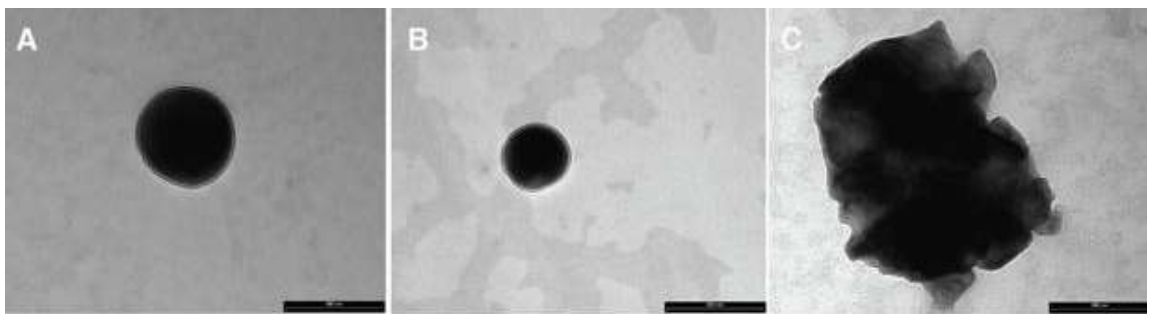


Figure 2

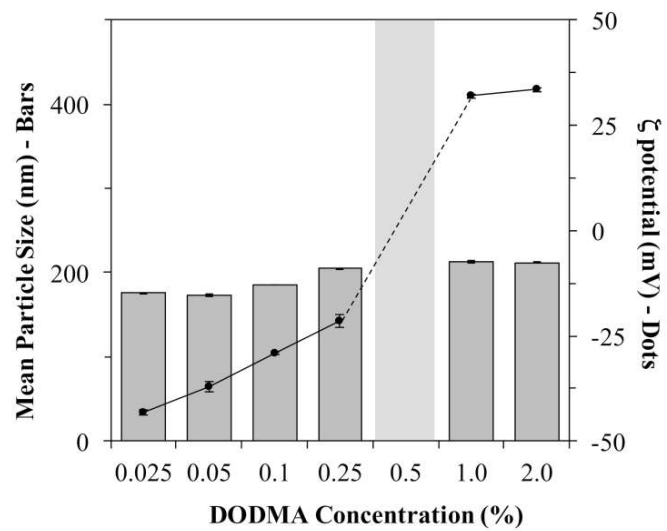


Figure 3

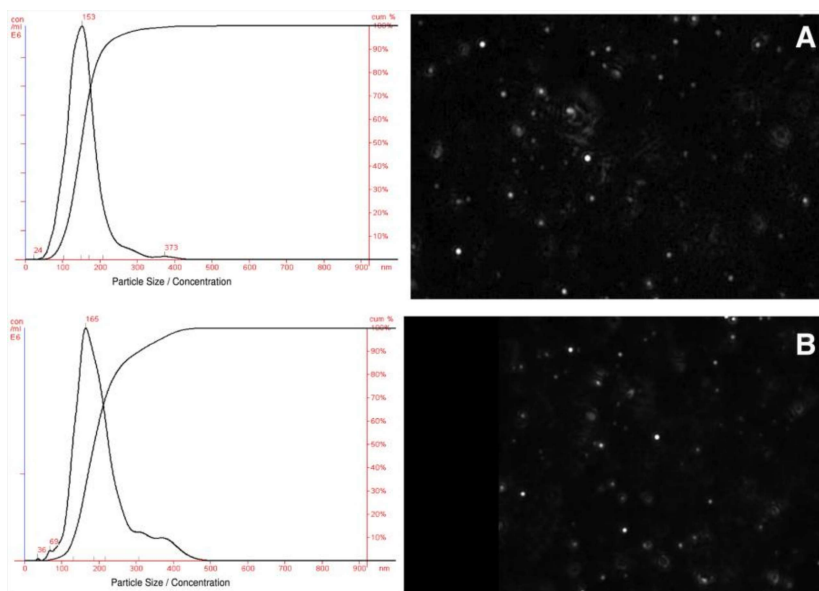


Figure 4

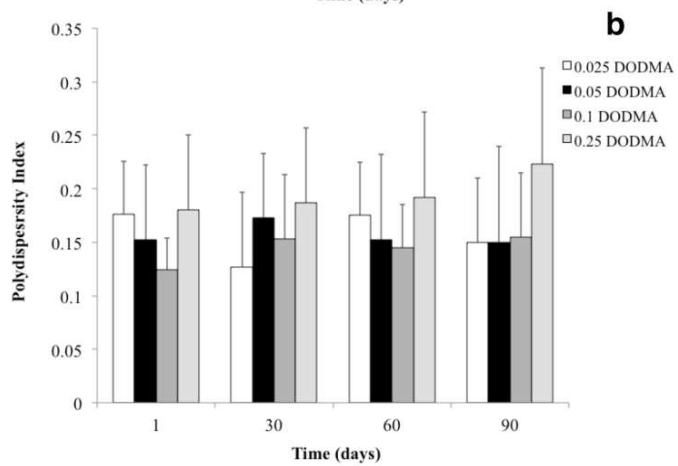
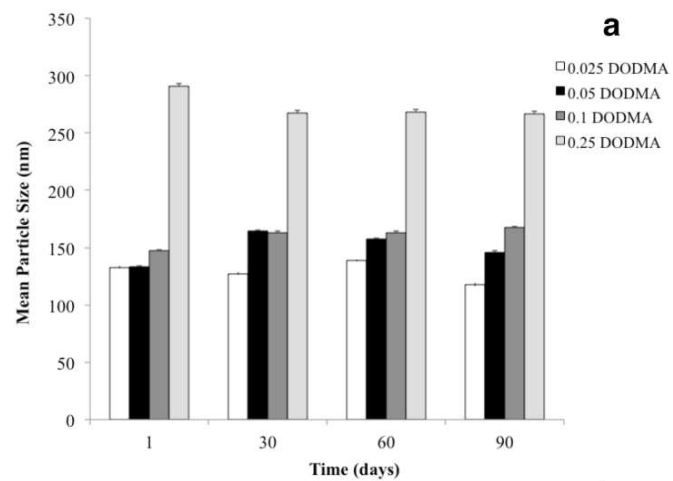


Figure 7

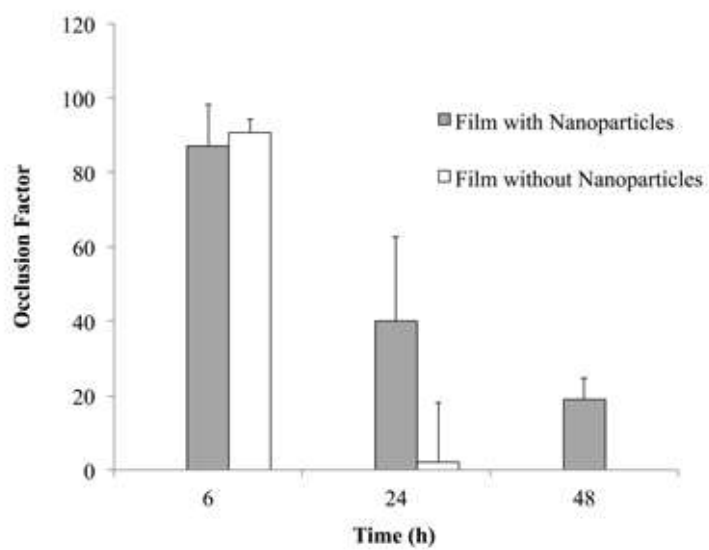
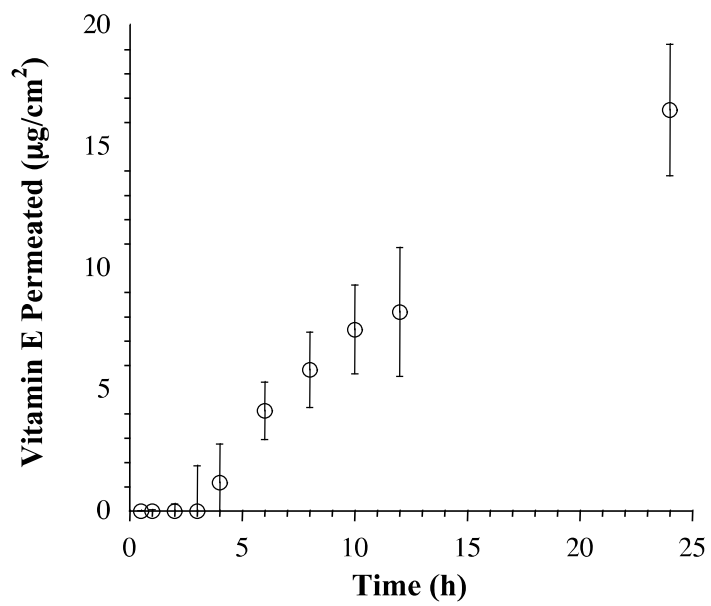


Figure 8



Conflict of interest statement

All authors declare no conflict of interest related to the subject of this paper.

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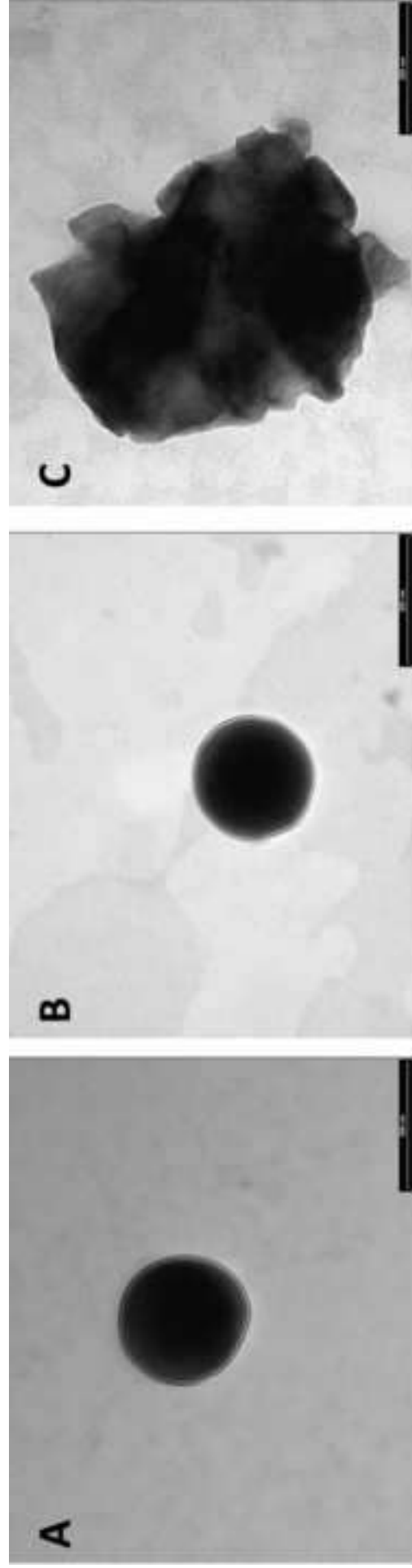
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Figure 1

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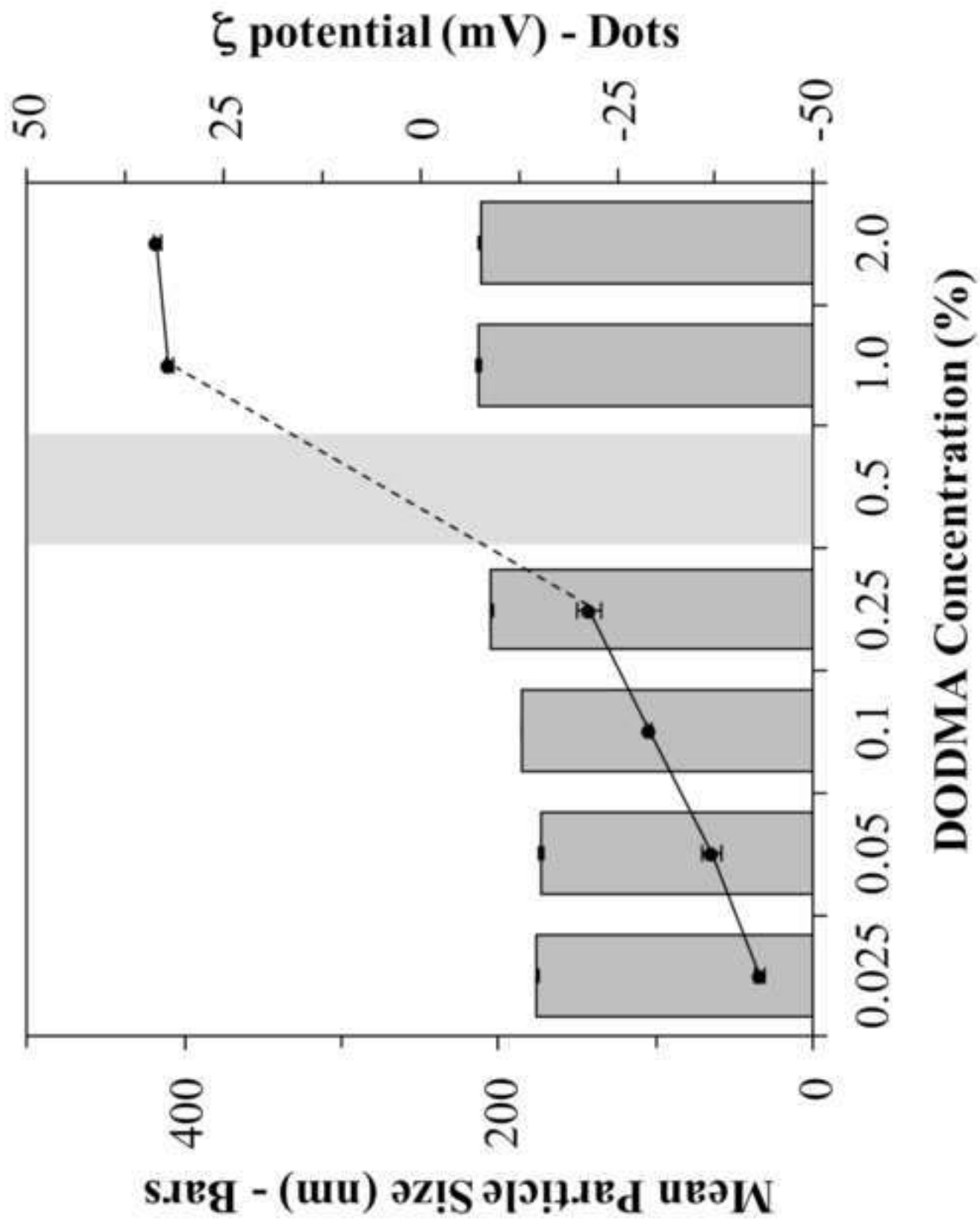


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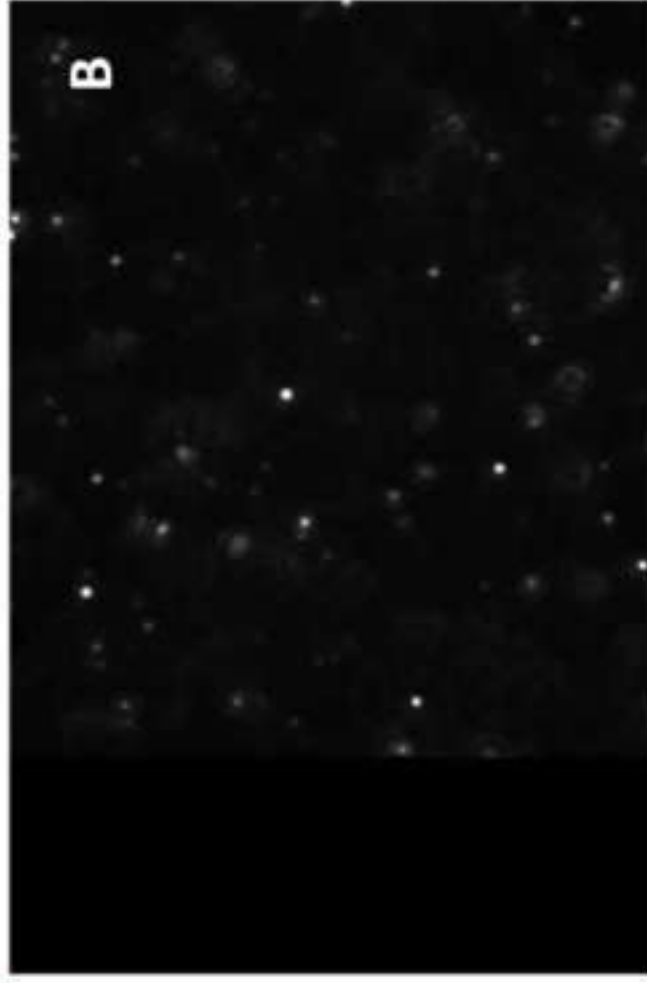
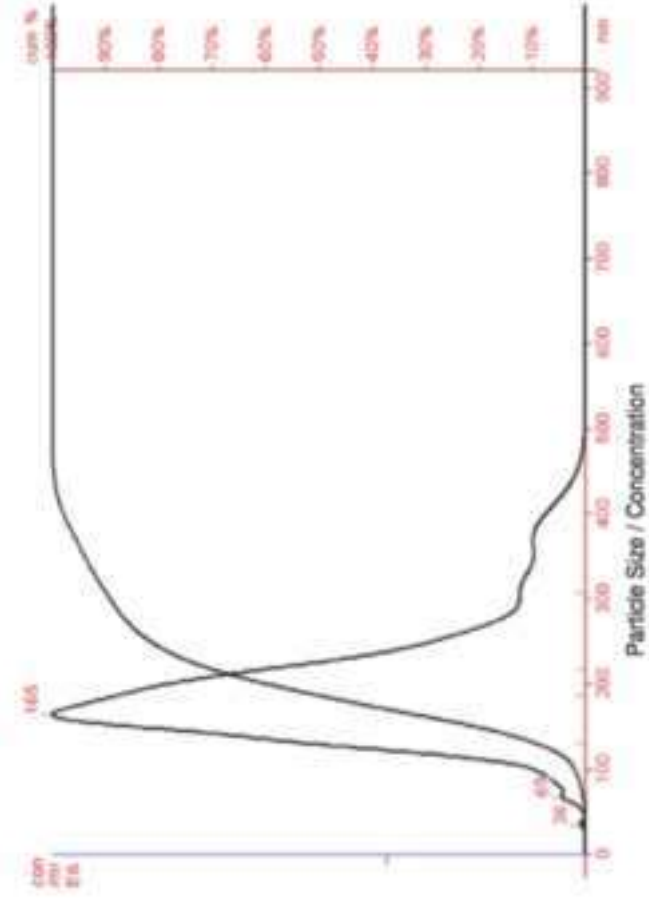
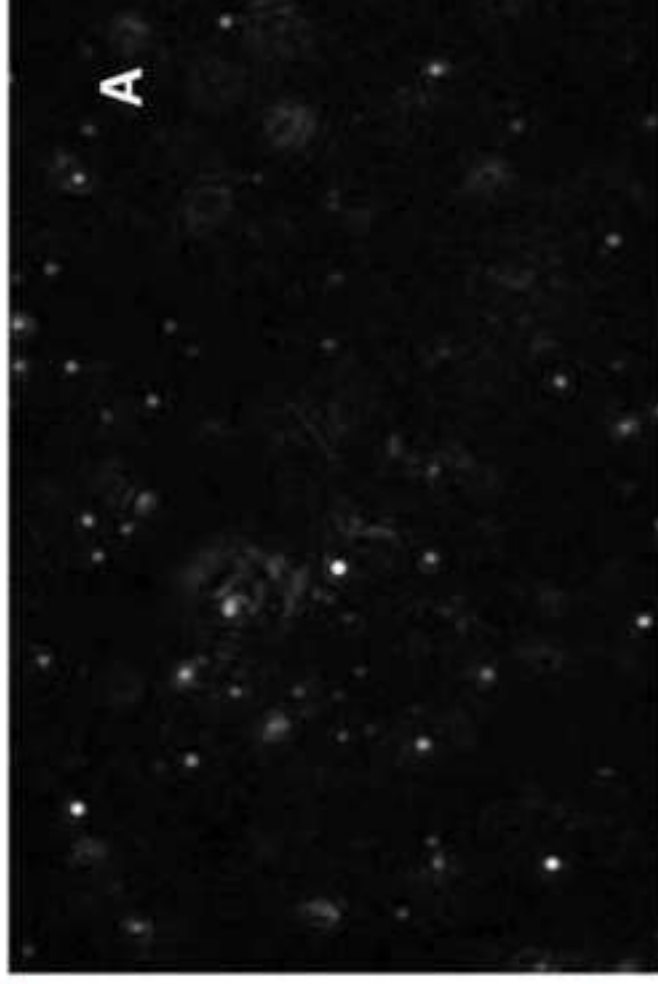
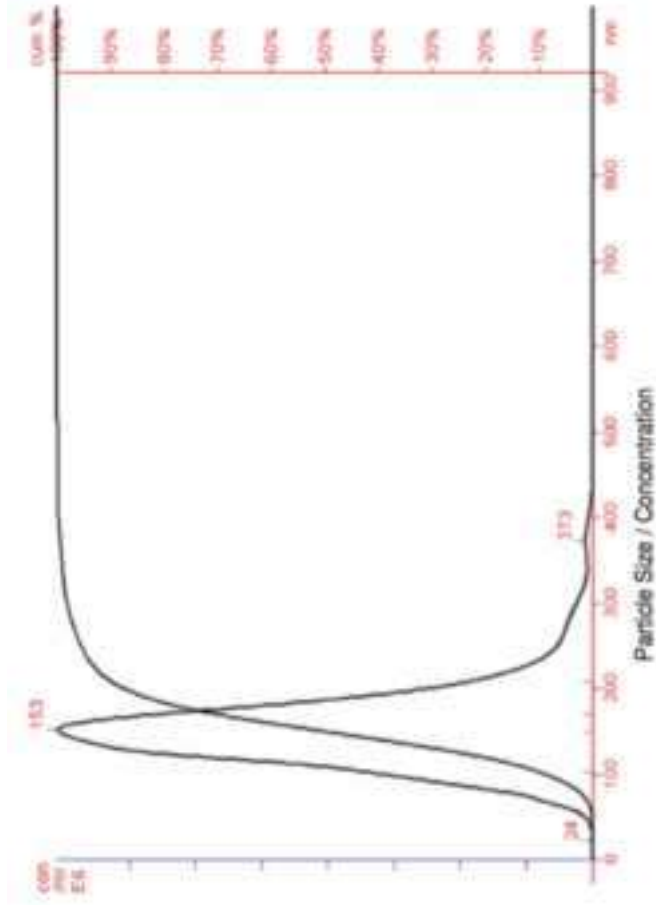


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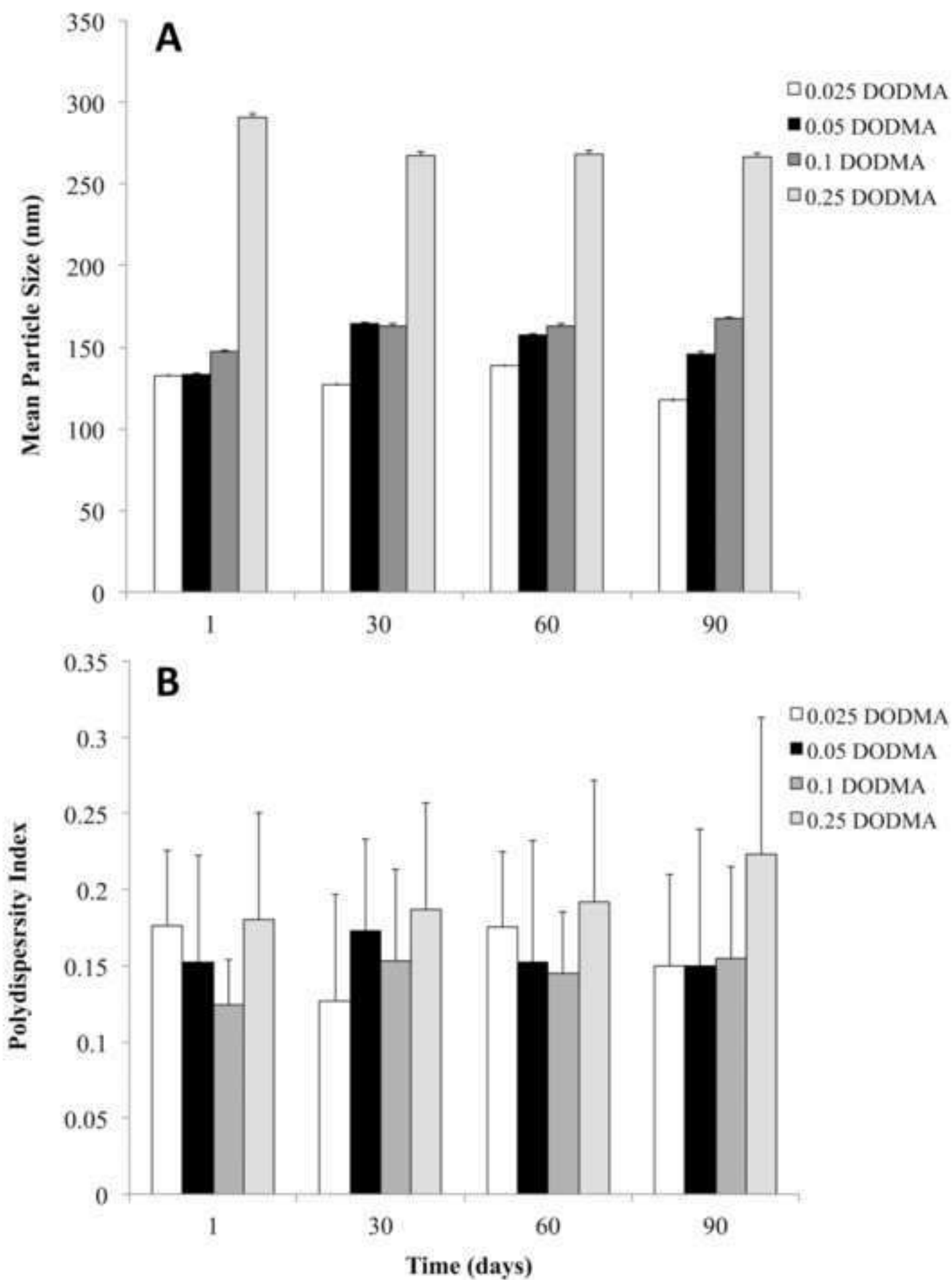


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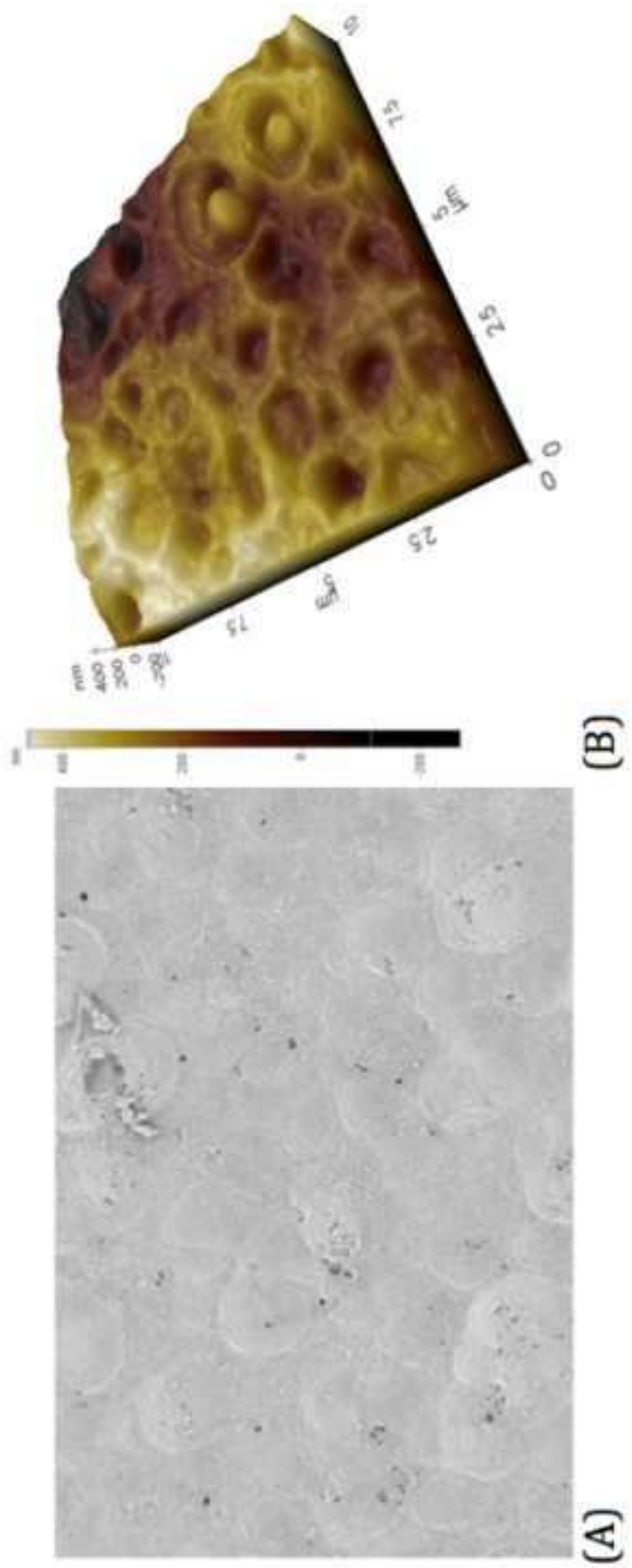


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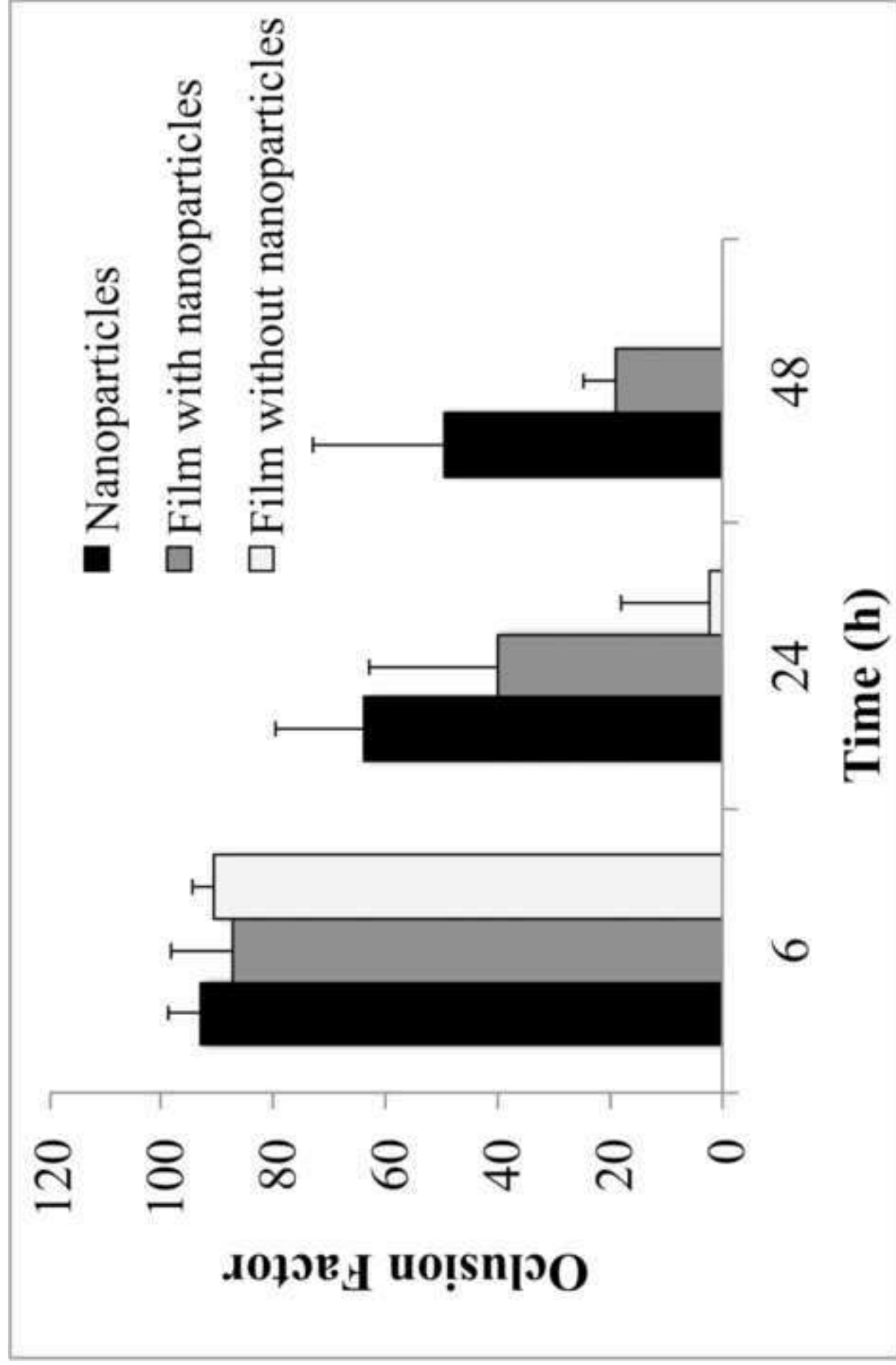
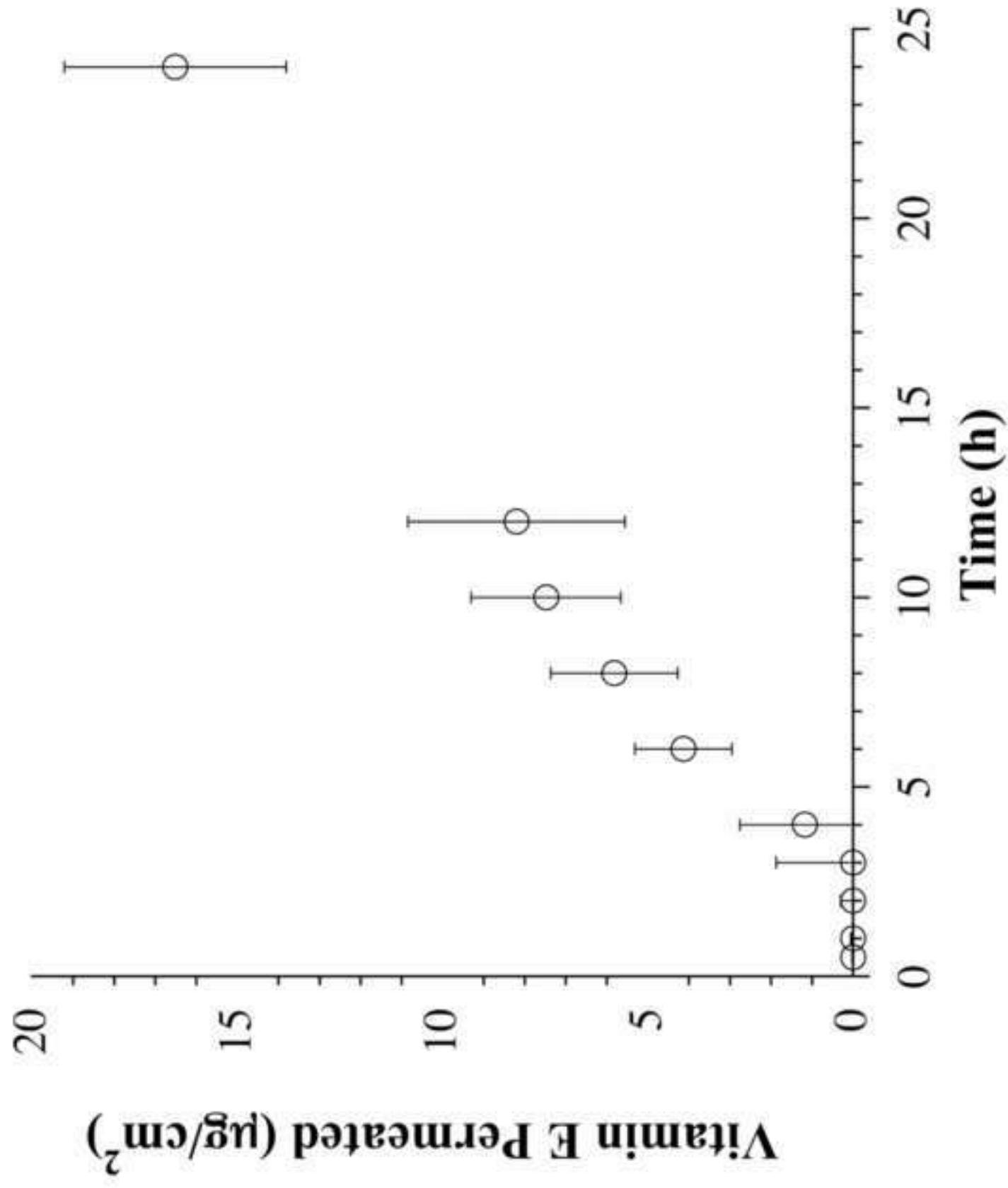


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1 **Table 1 - Percentage composition (% w/w) of Vitamin E acetate and *Aloe vera***
2 **loaded polymeric films.**

3

Components	Film
PVA (83,400 Da)	35.80
Sodium Hyaluronate	9.50
Sorbitol	27.90
PEO 12NF (1000 kDa)	13.50
Sodium Alginate	10.30
Vitamin E acetate in nanoparticles	2.00
<i>Aloe vera</i>	1.00

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2 **Table 2 - Volume distribution diameters (d_{10} , d_{50} and d_{90}), volume average**
3 **diameter ($D_{4.3}$) and Span values of nanoparticle formulations.**

4

Nanoparticle Formulations	d_{10} μm	d_{50} μm	d_{90} μm	$D_{4.3}$ μm	Span
0.025 DODMA	0.070	0.127	0.243	0.245 ± 0.030	1.359
0.05 DODMA	0.076	0.124	0.198	0.131 ± 0.010	0.982
0.1 DODMA	0.071	0.127	0.216	0.136 ± 0.010	1.146
0.25 DODMA	0.080	0.129	0.324	1.251 ± 0.025	1.895
0.5 DODMA			Agglomerates		
1.0 DODMA	0.091	0.262	17.766	8.117 ± 0.189	67.576
2.0 DODMA	0.098	0.280	18.001	8.250 ± 0.145	60.567

Table 3. Drug loading (DL) and encapsulation efficiency (EE) values of hyaluronic acid nanoparticle formulations.

Nanoparticle Formulations	DL	EE
	mg.ml⁻¹	%
0.025 DODMA	1.97 ± 0.05	99.98 ± 0.03
0.05 DODMA	1.94 ± 0.12	99.83 ± 0.05
0.1 DODMA	1.97 ± 0.03	99.99 ± 0.01
0.25 DODMA	1.96 ± 0.10	89.99 ± 1.00

Table 4: Characterization of vitamin E and Aloe vera loaded polymeric films (n=6).

Film Parameters	
Thickness (μm)	136.00 ± 0.23
Weight (mg/cm^2)	77.80 ± 0.14
Vitamin E (% w/w)	1.99 ± 0.10
Water Content (% w/w)	5.50 ± 0.06

Supplementary Material

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