RSC Advances

PAPER

View Article Online
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Cite this: *RSC Adv.*, 2016, **6**, 15929

Influence of vehicles on antioxidant efficacy in hair

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The UV radiation of sunlight is known to damage human hair, causing fibre degradation. Gallic acid (GA) was used as an active principle because of its antioxidant properties, which confer protection against free radicals. Encapsulation technologies, such as polymer-based (micro- and nano-spheres, capsules) and lipid-based systems (liposomes, emulsions), have been used to enhance and prolong the effectiveness of active ingredients. In this study, GA loaded in PCL microspheres (GA-Micro) and GA encapsulated in PC liposomes (GA-Lipo) were prepared to study their effectiveness as antioxidants when applied to human hair compared with treatment with free GA. The antioxidant effects of these structures were measured by the Bradford colorimetric assay and the thiobarbituric acid (TBA) assay and fluorescence spectroscopy using 2,7-dichlorodihydrofluorescein (DCFH) as the fluorescent probe. The penetration of GA into hair depending on the vehicle was also evaluated. Higher penetration of GA was found in hair fibres treated with GA encapsulated vehicles. The results of the antioxidant studies showed that the efficacy of GA increased when it was encapsulated in liposomes and microspheres, as well as when the studies were performed immediately after antioxidant treatment. Moreover, GA that was loaded into microspheres retained its antioxidant efficacy for one and two months after treatment, indicating that the microspheres could preserve the antioxidant effect of GA. Moreover, the spectrofluorometric method was found to be the best method to evaluate the efficacy of GA embedded in different vehicles.

Received 15th December 2015
Accepted 2nd February 2016

DOI: 10.1039/c5ra26815j

www.rsc.org/advances

Introduction

The UV components of sunlight are known to damage human hair, causing fibre degradation. UV-B attacks the melanin pigments and the protein fractions (keratin)[1] of hair, and UV-A produces free radical/reactive oxygen species (ROS) through the interactions of endogenous photosensitizers. The lipid and protein fractions play a major role in the structure and integrity of the hair fibre, protecting it against external agents. Therefore, damage to the protein and lipid fractions[2,3] can help us to evaluate the decomposition of the hair fibre.[4]

Gallic acid (GA) has been used as an active ingredient because of its anti-inflammatory, antifungal and antiviral properties and because of the antioxidant protection it provides against free radicals.[5,6] Its beneficial effects have led to its incorporation into the different vehicles that are applied to hair.

The efficacy of antioxidants depends on several parameters. The antioxidant should be an efficient radical scavenger. Furthermore, it should be able to penetrate into the hair shaft and interact with the melanin. An adequate carrier system is of the utmost importance. Encapsulation technologies with many biodegradable polymers (PCL, PMMA, PLGA) and lipids have been used to improve the solubility and long-term stability of the active ingredients in cosmetic formulations.[7,8] They have also been used to enhance and prolong the effectiveness of the active ingredients. Encapsulation technologies can be briefly categorized into polymer-based (micro- and nano-spheres, capsules) and lipid-based systems (liposomes, emulsions).

The encapsulation in suitable carrier systems, *i.e.*, liposomes, has been shown to enhance the penetration capacity of the active ingredients into hair and protect the antioxidants against oxidation.[9,10] They have received considerable attention because of their ability to trap both hydrophilic and hydrophobic active ingredients. However, the lipid-based systems have problems related to their long-term stability.[11,12] The polymer-based systems are generally more stable than the lipid-based systems.[13] The efficacy of an antioxidant in protecting hair is usually evaluated directly by measuring the strength, shine and colour maintenance after UV irradiation.[14] Colorimetric methods based on protein degradation and lipid peroxidation have been used to evaluate the effectiveness of antioxidants.[4] Because lipid and protein fractions play important roles in the hair structure, protein degradation and lipid peroxidation methods have been chosen. Additionally, a new method based on fluorescence spectroscopy has been developed. The Bradford colourimetric assay has been used to follow protein degradation; this method can quantify solubilized proteins and peptides.[6] Lipoperoxide formation due to the degradation of hair lipids by UV radiation was evaluated by thiobarbituric acid (TBA) assay.[6] Additionally, the antioxidant efficacy of GA encapsulated in different vehicles and applied to hair was studied by fluorescence spectroscopy using 2,7-dichlorodihydrofluorescein (DCFH) as the fluorescent

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probe.[15,16] The method used therein was a modification of a method used in cellular systems to study the formation of ROS in UVA irradiated cells and to evaluate the cellular oxidative stress.[17,18]

The purpose of this study was to prepare GA-loaded PCL microspheres (GA-Micro) and GA-PC liposomes (GA-Lipo) to study their effectiveness as an antioxidant when applied to human hair compared with free GA. GA-Micro and GA-Lipo were characterized by measuring their size distribution (mean particle size), polydispersity index (PI), Z-potential, and drug encapsulation efficiency (% EE). GA retention and penetration into the hair fibres were evaluated to determine the influence of vehicle administration. To determine the antioxidant treatment efficacy of GA encapsulated in PCL-microspheres, PC-liposomes and solubilized in ethanol/water solution, the antioxidant-treated hair fibres were subjected to UV irradiation. Photo-degradation of hair was then evaluated using the three assays mentioned above.

Materials and methods

Materials

For the preparation of the microspheres, poly(vinyl alcohol) (PVA; 87–89% hydrolysed, MW 31 000–50 000 Da) was used as the dispersant, and polycaprolactone (PCL; MW 45 000 Da) was used as the microsphere polymer. Both PVA and PCL were supplied by Sigma-Aldrich (Madrid, Spain). Ethanol was obtained from Merck (Darmstadt, Germany); isopropanol, from Carlo Erba (Milan, Italy); and dichloromethane, from Panreac (Barcelona, Spain). For liposome formation, soybean phosphatidylcholine (PC) (Lipoid S100; PC > 96%) was purchased from Lipoid GmbH (Ludwigshafen am Rhein, Germany). Gallic acid was supplied by Sigma-Aldrich (Madrid, Spain). For high-performance liquid chromatography with UV detector (HPLC-UV) analysis, methanol (HPLC grade) and purified water were used. Methanol (HPLC grade) was acquired from Merck (Darmstadt, Germany), and water was purified using an ultrapure water system, Milli-Q plus 185 (Millipore, Bedford, USA). Bovine Serum Albumin (BSA), thiobarbituric acid (TBA) and malondialdehyde (MDA) were acquired from Sigma (St. Louis, MO, USA). Methanol, hydrochloric acid (HCl; 37%), trichloroacetic acid (TCA) and sodium dodecyl sulphate (SDS) were supplied by Merck (Darmstadt, Germany).

Natural Caucasian brown hair tresses (20 cm in length) were purchased from De Meo Brothers Inc. (New York). Pantene® shampoo was supplied by Procter & Gamble Co. (Cincinnati, USA).

PCL microsphere preparation

PCL microspheres loaded with GA were prepared using the solvent evaporation method forming microemulsions ($W_1/O/W_2$ double emulsion). A total of 25 ml of a dispersion of 1 g of GA in monoalcohol (2% isopropanol/98% ethanol) was added dropwise to 15 ml of PCL (1 g) in dichloromethane. A simple emulsion (W_1/O) was generated by mechanical agitation (ULTRA-TURRAXT25, IKA) for 30 min at 24 000 rpm. Later, this simple emulsion was added to a continuous phase consisting of 100 ml of aqueous PVA solution (2%) and was emulsified for an additional 10 minutes at 24 000 rpm, resulting in a double emulsion ($W_1/O/W_2$). The preparation was carried out at 4 °C.[19] The mixture was maintained with agitation at 400 rpm for 20 h at room temperature, leading to the evaporation of the solvent and, consequently, the formation of the microspheres.

Liposome preparation

Liposomes were prepared using the film hydration method.[20] Three grams of Lipoid® S100 (PC), as the lipid, was weighed and dissolved in 30 ml of chloroform. The mixture was rotary evaporated into a thin lipid film, which was rehydrated with 100 ml of a 2% aqueous solution of GA producing multilamellar vesicles (MLV).

Microspheres and liposomes characterization

The hydrodynamic diameter (HD) and polydispersity index (PI) of the aggregates were measured using the Zetasizer nano ZS (Malvern Instruments, UK). This equipment employs the dynamic light scattering (DLS) technique to determine particle sizes between 0.6 nm and 6 μm. DLS measures the Brownian motion of the particles and correlates this phenomenon to their particle size.[21] To minimize multiple scattering, non-invasive back scatter technology (NIBS) was used. With this technology, the scattering is detected at an angle of 173°. Therefore, concentrated samples can be measured without any modification in the case of our systems. The measurements were performed at room temperature with DTS1060C-Clear disposable zeta cell, and water was used as the dispersant. The results were reported as the percentage of scattering intensity. Z-potential was also measured. The data were collected and analysed using the DTS (Dispersion Technology Software) program provided by Malvern Instruments Ltd.

Drug encapsulation efficiency (EE%)

To quantify the encapsulated GA, 1 ml of each of the PCL microsphere and the liposome formulation were precipitated and separated from the supernatant by centrifugation at 14 000 rpm for 15 min using a Biocen 22 R centrifuge (Orto Alersa, Spain). After separation, the supernatant was retained. The initial PCL microspheres, liposomes and both supernatants were diluted in methanol and analysed by HPLC. The encapsulation efficacy of GA in the microspheres and in the liposomes was determined by measuring the amount of the active ingredient present in the entire solution, as well as in the supernatant, obtained from a GA calibration curve.

The percentage of encapsulation efficiency (EE%) in the microspheres and in the liposomes is given by:

$$\mathrm{EE\%} = [\mathrm{drug(encapsulated)/drug(total)}] \times 100$$

HPLC analysis

The concentration of GA in the different samples and the EE% were determined by using a VWR-Hitachi LaChrom Elite HPLC system (Darmstadt, Germany). The equipment consisted of an

L-2130 pump, an L-2200 autosampler and an L-400 UV detector. The system was operated using Merck EZChrom Elite v 3.1.3 software. Chromatographic separation was performed at room temperature using a LiChrocart 125-4/Lichrosorb RP-18 (5 mm) column (Darmstadt, Germany). The mobile phase was 90% methanol/10% water + 0.7% H_3PO_4 flowing at a rate of 1.0 ml min^{-1}. A total of 20 μl of sample or calibration standard was injected into the column and eluted with the mobile phase. Detection was carried out by monitoring the absorbance at 280 nm. The calibration curve exhibited a linear behaviour (>0.99%) over the concentration range of 0.104–130 μg ml^{-1}.

Hair treatments

The fibre samples were all Caucasian brown hair. Hair swatches of 2 g weight were used to carry out protein degradation, lipoperoxidation, fluorescence spectroscopy tests and hair penetration evaluation. The hair swatches were initially washed using 40 mg of Pantene® shampoo, and then rinsed with 4 × 250 ml of distilled water to remove any traces of cosmetic or dirt. Hair strands weighing approximately 2 g were treated with 1 ml of each of the different formulations containing 1% GA (microspheres, liposomes and ethanolic-aqueous solution) or placebo (vehicles without GA) twice a day for 5 days. Before each subsequent application, the hair was washed as in the first wash. During the experimentation the hair samples were kept in a temperature and humidity-controlled room (23 ± 2 °C, 50 ± 5% RH) and preserved to the light.

Antioxidant hair penetration

The total GA retained in the hair and the GA that penetrated into the fibre, depending on the vehicle used, were evaluated using the following procedure. To remove the external GA from the hair surface, a small strand (approximately 40 mg) of each hair treated with GA encapsulated in different vehicles were washed three times with 10 ml of 0.1% SDS with ultrasonication for 1 min. And then washed three times with 15 ml of water using the same conditions.[22] The samples were then dried at room temperature.

To determine the total and the internalized amount of GA in the hair fibres, washed and non-washed samples were cut into snippets of 1–3 mm. To ensure sample homogeneity, at least of 20–30 mg of hair were subjected to the above procedure. To extract the GA from the hair samples, the washed and non-washed fibres were subjected to ultrasonication for 10 min in methanol (10 ml). The extracted samples were analysed by HPLC (as previously described) to determine the total amount of GA that was retained in the hair and the amount of GA that penetrated into the hair matrix. This study was performed in triplicate for each hair sample.

Exposure to artificial UV radiation

Each swatch was divided into two parts (0 and 72 h): one part was not irradiated (0 h), whereas the other part was subjected to UV irradiation for 72 hours on the SUNTEST CPS + (250 W m^{-2} equivalents to 1.5 J min^{-1} cm^{-2}). During evaluation by fluorescence spectroscopy, the time of exposure to UV radiation was 2 hours.

Protein degradation test

The Bradford colorimetric assay was used to test protein degradation. This assay quantifies dissolved proteins and peptides. It is based on the formation of a complex between the dye, Brilliant Blue G, and the proteins in solution, leading to an increase in absorption at 595 nm that is proportional to the amount of protein in the solution (4). BSA was used as a standard to calculate the amount of protein.

Approximately 100 mg of hair in different swatches (treated and exposed to UV irradiation or untreated) were weighed. Dissolution of hair proteins and peptides was carried out in 2 ml of 2% aqueous SDS solution, which was sonicated using a Labsonic 1510 device (B. Braun, Melsungen, Germany) for 5 h, at 45 °C. The hair extracts were then diluted down to 0.01% SDS concentration, and the Bradford colorimetric assay was performed. This assay was performed in triplicate for each hair swatch. Tests with untreated hair (only washed with shampoo) were also performed.

Lipid peroxidation measurements

Lipid peroxidation refers, in our case, to an oxidative degradation of the lipids in hair by UV irradiation. To evaluate lipoperoxidation, the thiobarbituric acid reactive substances (TBARS) assay[4] was performed. It is based on the reaction of MDA (one of the products formed by the decomposition of the lipoperoxided compounds) with TBA to form a coloured complex (MDA:TBA).

Approximately 500 mg of different hair samples were weighed. Then, the lipids from each of the different hair sample were extracted with methanol (500 mg hair/10 ml methanol) and sonicated for 15 min at room temperature. Next, the hair extracts were dried under an N_2 current and diluted again in 1.5 ml of methanol.

The results are expressed as malonaldehyde bis(dimethylacetal) equivalents (μM MDA) using a standard curve for the pure MDA–TBA complex. The calibration curve was obtained by using different concentrations of MDA (0.5–30 μM). The absorbance values obtained from the hair samples allowed us to determine the amount of oxidized lipid in each sample. Here, the absorbance values from each of the samples were extrapolated using the equation from the calibration curve. The results were expressed as the number of equivalents of MDA peroxides formed (in μM concentrations). Ultimately, the amount of MDA peroxides formed for each mg of hair was found to relate to the initial amount of hair.

Evaluation of antioxidant efficacy by fluorescence spectroscopy

To evaluate the antioxidant efficacy of the application on the hair of GA encapsulated in the two different vehicles (GA-Lipo and GA-Micro) and as an aqueous-ethanolic solution (GA-Aqu), DCFH was used as the fluorescent probe. DCFH is a non-fluorescent molecule that in contact with an oxidizing

agent, as free radicals generated by UV-radiation, produces 2,7-dichlorofluorescein (DCF) (Fig. 1). DCF is a fluorescent compound ($\lambda_{excitation}$ = 488 nm; $\lambda_{emission}$ = 522 nm) that was initially thought to be useful as a specific indicator for the quantitation of reactive oxygen species (ROS).

The method used here is based on that is based in the method described by Venkatachari.[23] The DCFH solution was prepared by first cleaving 2′,7′-dichlorofluorescin diacetate (DCFH-DA) in 0.01 N NaOH solution according to the following procedure. Briefly, 6 ml of an ethanolic solution of 1 mM DCFH-DA was hydrolysed to DCFH with 24 ml of 0.01 N NaOH solution. The reaction mixture was placed in the dark for 30 minutes at room temperature, and then neutralized with 60 ml of phosphate buffered saline (PBS). The solution was kept on ice in the dark until further use.

Approximately 500 mg of hair subjected to different treatments and exposure to UV radiation, or untreated hair, were weighed. A total of 10 ml of the DCFH solution was added to the different hair samples, which were incubated at 37 °C for 1 hour and 30 minutes in the dark. After incubation, the solutions were collected to measure the DCF formed due to the generation of ROS in the hair when exposed to UV radiation. The DCF was detected with a Shimadzu RF-540 spectrofluorometer.

Statistical analysis

The results obtained in the different experiments are expressed as the mean ± standard deviation (SD) for three determinations. As regards to the statistical significance of the results obtained for each vehicle used and experiment carried out, analysis of variance (ANOVA) and a Kruskal–Wallis test have been applied. The software used was the Statgraphics® plus 5. A p value of less than 0.1 was considered significant.

Results and discussion

Three formulations were prepared with the aim to obtain 1% GA encapsulated in PC liposomes and PCL microspheres, and solubilized in ethanol/water solution (1 : 1). Following the experimental steps described previously, the three formulations, GA-Lipo, GA-Micro and GA-Aqu, were obtained. The GA concentrations were determined for all formulations by HPLC analysis. Similar concentrations of approximately 1.1% GA were obtained for all the three samples (see Table 1). The GA formulations were characterized using DLS and HPLC to determine particle size distribution, zeta potential and drug encapsulation efficiency (EE%) (Table 1). In the following table, the values obtained from the characterization of GA-Micro and GA-Lipo are shown.

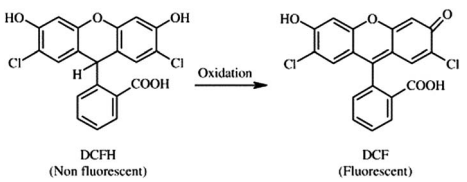

Fig. 1 DCF formation by oxidation of DCFH.

The percentage of GA concentration in the three formulations are very similar, 1.08 ± 0.09% for GA-Lipo, 1.15 ± 0.08% for GA-Micro and 1.15 ± 0.02% for GA-Aqu. The EE% was 34.8% for GA-Micro and 20.9% for GA-Lipo. These values indicated a higher encapsulation of GA in the microspheres than in the liposomes.

The results obtained by DLS for GA-Lipo and GA-Micro showed mean particle sizes of 320.75 ± 32.28 nm (PI 0.630) and 2096.63 ± 122.78 nm (PI 0.356), respectively. The results indicated a larger diameter for GA-Micro than for GA-Lipo. These results were expected because the size for liposomes generally ranges from 30 nm to several micrometers,[24] whereas that for the microspheres made up with PCL ranges from 1 to 20 μm (mostly in the range of 5–10 μm).[25] The larger size of the microspheres was in accordance with the higher % EE of GA in the microspheres. Moreover, the polydispersity index shows greater homogeneity for GA-Micro (0.356) than for GA-Lipo (0.630).

The Z-potential values obtained were +8.57 mV in the case of GA-Lipo and −0.97 mV in the case of GA-Micro. Because the hair fibres are negatively charged,[26] GA-Lipo appears to show better interaction with hair, but the low negative charge on GA-Micro may not provide a great impediment to interact with the hair fibres either. Moreover, the higher the Z-potential in magnitude (it does not matter whether the zeta potential is + or −), the better the stability of the particles due to repulsion. At lower values of Z-potential, the repulsion between particles is minor; therefore, they tend to flocculate. Therefore, these results indicated a higher stability for GA-Lipo compared with GA-Micro.

In the hair penetration evaluation studies, the total amount of GA retained (both inside and outside of the hair samples) and the amount of GA that penetrated into the hair matrix were quantified. The results obtained are included in the following table (Table 2). In addition, a graphical representation of the percentage of GA penetration with respect to the total amount applied to the hair is plotted in Fig. 2.

The results of the hair penetration studies indicated that the hair treated with the aqueous solution of GA (GA-Aqu) retained more GA than the hairs treated with the other formulations, especially, the differences are significant with hair treated with liposomes. The microspheres also significantly helped to retain GA to a greater extent than the liposomes. This is in contrast with the Z-potential results that indicated the possibility of a better interaction between GA-Lipo and the hair fibres. However, it is important to note that a higher amount of GA was found inside the fibres when the two vehicles, liposomes and microspheres, were applied. Statistically differences are found between penetration of GA vehiculized in microspheres and solubilized with ethanolic-aqueous solution.

To evaluate the efficacy of the GA encapsulated in the different vehicles as an antioxidant, three types of tests were carried out: protein degradation, lipid peroxidation and fluorescence spectroscopy. Efficacy was evaluated at different times after treatment. This was planned to determine the possible

Table 1 Average values (±SD) from the characterization of GA-Lipo, GA-Micro and GA-Aqu

Sample	Conc (%)	*D* (nm)	PI	Z-Pot (mV)	% EE (%)
GA-Lipo	1.08 ± 0.09	320.75 ± 32.28	0.630	8.57	20.9
mA-Micro	1.15 ± 0.08	2096.63 ± 122.78	0.356	−0.97	34.8
GA-Aqu	1.15 ± 0.02	—	—	—	—

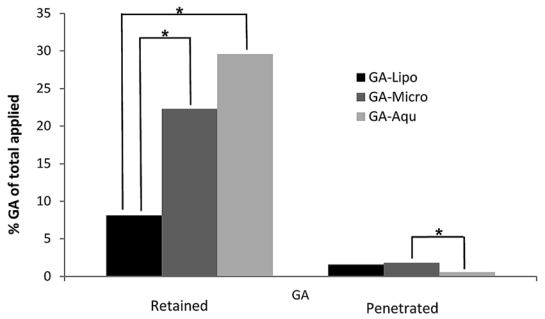

Fig. 2 Graphical representation of the GA retained and the GA that penetrated with respect to the total amount of GA in the three formulations that were applied to the hair. *$p > 0.1$.

preservation of the antioxidant effect of GA due to encapsulation.

Because proteins constitute of a major part of hair (88%), and UV radiation leads to loss of protein,[27] a protein degradation test is a good way to study the efficacy of an antioxidant against damage due to UV radiation. Although it is not easy to evaluate lipid modification because of the low amount of lipids in the hair fibres (2.3%),[28] a lipid peroxidation test is also an efficient method to study the damage produced by UV radiation, and, hence, the efficiency of an antioxidant applied to hair. Fluorescence spectroscopy using DCFH as the fluorescent probe is another tool for evaluating the generation of ROS due to UV irradiation in hair.

The results of the three tests immediately after the application of GA vehiculized in different vehicles to the hair samples, irradiated or not, are shown in Table 3. The graphical representation of the percentage of oxidation increase in the hair samples, treated with the different formulations of GA and submitted to UV radiation, which was evaluated using the aforementioned techniques, are exhibited in Fig. 3. The percentages are relative to 100% oxidation of untreated hair samples.

It can be pointed out the antioxidant efficacy of all formulations containing GA evaluated by the three methodologies. Moreover, the results show that GA-Lipo and GA-Micro provide better protection against UV radiation than free GA when evaluated by protein solubilization and fluorescence spectroscopy. The higher antioxidant protection observed for the GA-encapsulated formulations can be attributed to the higher amount of the antioxidant present inside the fibres due to the penetration promoted by the liposomes and microspheres. In contrast, the lipid peroxidation values indicated that better protection was provided by GA-Aqu. However, the different values obtained for the non-irradiated samples indicated that lipid peroxidation could affect not only the small lipid fraction in the hair but also the lipid components of the liposomes and microspheres, which can otherwise mask the lipid peroxidation of the components of the hair fibre. Therefore, the lipid peroxidation test was discarded and not used for further evaluation.

Protein degradation and fluorescence spectroscopy studies of GA-Lipo and GA-Micro one and two months after application to hair were performed to evaluate the stability of GA and the antioxidant efficacy of GA in liposomes and microspheres over time. Free GA was not evaluated over time because it demonstrated poor results in terms of its antioxidant efficacy just before the application. A comparison of the results obtained from the protein degradation and spectrofluorometry tests for GA-Lipo- and GA-Micro-treated and untreated hair samples immediately after application and one and two months after application are presented in the following table (Table 4):

The graphical representations of the values obtained from the protein degradation and fluorescence spectroscopy tests immediately after the application of GA to hair and after one and two months are presented in Fig. 4.

The results obtained from the protein degradation and fluorescence spectroscopy tests, which were carried out immediately after GA application and one and two months later, showed a similar trend. When GA was loaded into the liposomes (GA-Lipo), most of the antioxidant effect achieved immediately after applications was lost with time; there was less antioxidant effect after 1 month and 2 months. However, the effectiveness of GA-Micro was clearly maintained over time. This difference in the antioxidant efficacy of GA between encapsulation in liposomes and in microspheres (especially statistically significant in the spectrofluorometric results) can be attributed to the difference in their EE%. GA-Micro has a higher % EE than GA-Lipo. Liposomes are known to have lower stability over time,[11,12] whereas polymer based

Table 2 Average values (±SD) obtained in the evaluation of hair penetration

Sample	Total applied (μg GA per mg hair)	Total amount GA retained (μg GA per mg hair)	% GA retained of total applied	Internal amount GA penetrated (μg GA per mg hair)	% penetrated of amount retained	% penetrated of total applied
GA-Lipo	5.40	0.44 ± 0.14	8.11 ± 2.53	0.08 ± 0.05	22.74 ± 19.42	1.57 ± 0.85
mA-Micro	5.75	1.28 ± 0.55	22.28 ± 9.48	0.10 ± 0.05	9.58 ± 6.04	1.77 ± 0.83
GA-Aqu	5.75	1.70 ± 0.57	29.55 ± 9.93	0.03 ± 0.02	2.18 ± 1.23	0.57 ± 0.26

Table 3 Average values (±SD) obtained in the protein degradation, lipid peroxidation and fluorescence spectroscopy tests carried out immediately after GA application to the hair and after 0 h (non-irradiated), 2 h or 72 h of UV irradiation

		GA-Lipo	GA-Micro	GA-Aqu	Untreated
Protein degradation [μg protein per mg hair]	0 h UV	36.50 ± 5.51	38.91 ± 3.23	41.59 ± 6.86	33.69 ± 6.57
	72 h UV	38.91 ± 3.49	40.99 ± 9.21	46.76 ± 5.00	53.64 ± 9.52
	% increase	7.28 ± 6.61	4.67 ± 16.85	13.27 ± 9.24	59.85 ± 11.86
	Relative to 100% untreated	11.52 ± 10.75	9.12 ± 29.83	20.96 ± 10.89	100 ± 0.00
Fluorescence spectroscopy (intensity (u.a.))	0 h UV	22.08 ± 2.98	18.18 ± 3.39	17.65 ± 0.80	45.75 ± 3.19
	2 h UV	30.91 ± 7.54	29.23 ± 3.49	32.99 ± 3.07	102.27 ± 1.26
	% increase	39.90 ± 30.03	66.20 ± 44.22	89.90 ± 16.42	124.21 ± 15.16
	Relative to 100% untreated	31.59 ± 21.46	55.34 ± 38.38	71.82 ± 23.13	100 ± 0.00
Lipid peroxidation [μM MDA per mg hair]	0 h UV	3.48 ± 2.05	1.26 ± 0.73	2.27 ± 0.95	0.41 ± 0.36
	72 h UV	8.54 ± 2.87	4.01 ± 0.94	3.38 ± 1.72	3.38 ± 2.42
	% increase	193.29 ± 153.92	271.97 ± 139.69	45.85 ± 27.57	795.20 ± 144.35
	Relative to 100% untreated	22.45 ± 16.16	33.16 ± 13.94	6.02 ± 3.85	100 ± 0.00

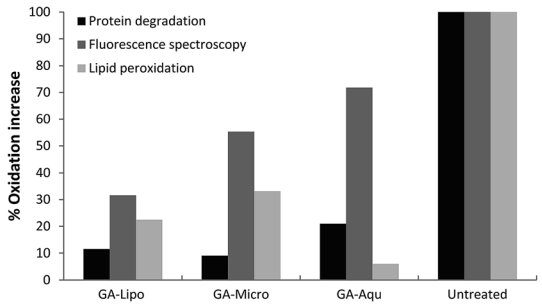

Fig. 3 Comparison of % increase in oxidation from the different techniques studied immediately after the application of GA formulations, relative to 100% oxidation of the untreated hair samples.

microspheres are usually more stable. Moreover, the slightly higher penetration of GA into the hair fibres when it is encapsulated in microspheres can also support its greater antioxidant efficacy over time. These results were indicative of better protection of GA against degradation when it is encapsulated in microspheres than in liposomes at least two months after the application of GA formulations to hair samples. Therefore, microspheres are a better candidate to encapsulate GA for application to hair over time.

In this work, three assays were performed to determine the antioxidant properties of GA encapsulated in different ways. The TBARS assay is a well-recognized and established method for quantifying lipid peroxidation of a substrate subjected to UV radiation. Unfortunately, in our case, there was interference with

Table 4 Values obtained in the protein degradation and fluorescence spectroscopy tests carried out immediately after GA application to hair and one and two months thereafter

			GA-Lipo	GA-Micro	Non treat
Protein degradation [μg prot per mg hair]	Just applied	0 h UV	36.50 ± 5.51	38.91 ± 3.23	33.69 ± 6.57
		72 h UV	38.91 ± 3.49	40.99 ± 9.21	53.64 ± 9.52
		% increase	7.28 ± 6.61	4.67 ± 16.85	59.85 ± 11.86
		Relative to 100% non treat	11.52 ± 10.75	9.12 ± 29.83	100 ± 0.00
	1 month after	0 h UV	33.72 ± 4.93	42.96 ± 18.30	44.05 ± 3.30
		72 h UV	47.35 ± 3.49	46.88 ± 9.41	57.85 ± 19.29
		% increase	39.04 ± 18.84	16.84 ± 30.64	29.98 ± 34.56
		Relative to 100% non treat	146.08 ± 100.54	24.08 ± 22.04	100 ± 0.00
	2 months after	0 h UV	42.91 ± 6.32	45.63 ± 2.13	35.40 ± 16.89
		72 h UV	45.12 ± 5.37	45.28 ± 8.76	43.54 ± 10.46
		% increase	5.37 ± 3.02	−3.42 ± 17.76	37.03 ± 47.31
		Relative to 100% non treat	30.28 ± 37.82	−55.83 ± 104.69	100.00
Fluorescence spectroscopy (intensity (u.a.))	Just applied	0 h UV	22.08 ± 2.98	18.18 ± 3.39	45.75 ± 3.19
		2 h UV	30.91 ± 7.54	29.23 ± 3.49	102.27 ± 1.26
		% increase	39.90 ± 30.03	66.20 ± 44.22	124.21 ± 15.16
		Relative to 100% non treat	31.59 ± 21.46	55.34 ± 38.38	100 ± 0.00
	1 month after	0 h UV	22.56 ± 4.52	20.32 ± 0.90	26.07 ± 8.15
		2 h UV	78.27 ± 23.68	27.05 ± 2.50	102.32 ± 18.97
		% increase	243.27 ± 36.14	33.50 ± 18.22	300.72 ± 52.57
		Relative to 100% non treat	83.22 ± 26.56	10.78 ± 4.18	100 ± 0.00
	2 months after	0 h UV	18.91 ± 3.90	17.81 ± 2.93	21.08 ± 2.17
		2 h UV	56.39 ± 6.55	23.67 ± 6.70	75.63 ± 4.43
		% increase	200.97 ± 27.39	31.62 ± 15.97	261.81 ± 58.27
		Relative to 100% non treat	79.90 ± 28.24	11.69 ± 3.50	100 ± 0.00

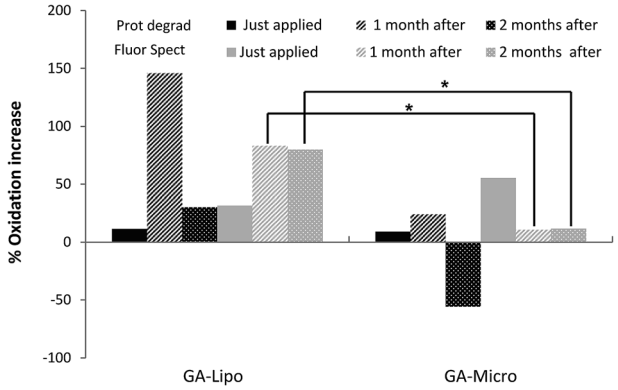

Fig. 4 Comparison of the % increase in oxidation obtained from the protein degradation and fluorescence spectroscopy studies immediately after the application of GA to hair and after one and two months, related to 100% oxidation of untreated hair samples.

the lipidic matrix of the liposomes, which caused significant standard deviations in the results. Other studies were carried out using the Bradford test; this assay is useful in the evaluation of protein degradation due to oxidation by UV radiation. As a consequence, the effectiveness of the antioxidant could be evaluated. This method is simple and faster than the other methods used to evaluate protein degradation but is also subject to interference from the non-protein components of biological samples.[29] In our work, the results showed an significant standard deviation indicating that this was not the best method to evaluate the effectiveness of GA, loaded in different vehicles, as an antioxidant. The last method used to evaluate the antioxidant efficacy of GA in different vehicles was a modification of a method that was used to study the formation of ROS in UVA irradiated cells[30,31] and to evaluate cellular oxidative stress.[17,18] This spectrofluorometric method is based on the oxidation of DCFH to DCF in the presence of an oxidizing agent. This method adapted for our samples is rapid and has a high reproducibility, as indicated by the low standard deviation in our results.

Conclusions

In summary, these results indicate that microspheres could preserve the antioxidant efficacy of GA over a longer period of time, which was due in part to their higher penetration ability. Moreover, the spectrofluorometric method was found to be the best method to evaluate the efficacy of GA embedded in the different vehicles. Future work will be based on the vehiculization of other antioxidants in microspheres to demonstrate their efficacy along the time as it was the case of GA. This approach could serve to verify the use of encapsulated antioxidants to be released into the hair. The present study opens new prospects for the development of antioxidant formulations able to increase hair protection.

Acknowledgements

The authors wish to thank the Ministerio de Economia y Competitividad (Spanish National Project CTQ2013-44998-P) and the Generalitat de Catalunya (2014 SGR 1325).

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