Anticancer Activity of a Hydrogel Containing Folic Acid Towards MCF-7 and MDA-MB-231 Cells

SONIA TROMBINO, TERESA FERRARELLI, MICHELE PELLEGRINO, EMILIA RICCHIO, LOREDANA MAURO, SEBASTIANO ANDÒ, NEVIO PICCI and ROBERTA CASSANO

Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Cosenza, Italy

Abstract. *Aim: The aim of the present study was to prepare a hydrogel, based on ellagic acid and glycine, embedded with folic acid, as a subcutaneous implant for the treatment of breast cancer. The function of folic acid is to selectively and actively target tumor cells which are well-known to overexpress folic acid receptors on their surface. Materials and Methods: A pro-drug based on L-glycine and ellagic acid, was functionalized with a polymerizable group and loaded with folic acid to make it more natural, non-toxic, compatible and specific for the site of action. Cytotoxicity against MCF-7 cells was also evaluated. Release studies of folic acid were conducted on aliquots of hydrogel at different pH (6.2 and 7.4) and time-points (1, 6, 12 and 24 h) using a shaking water bath at 37˚C (body temperature). Results: Our results show that folic acid release by the hydrogel is characterized by a slow kinetic release, especially at pH 6.2. Moreover, it was evidenced that the exposure of human breast cancer cells to ellagic acid-based hydrogel containing folic acid significantly reduced cell viability.*

The use of polymeric materials in the pharmaceutical fields has enabled for creation of various drug delivery systems based on a wide range of biocompatible polymers (1-4) with hydrophilic structures and different physicochemical properties (5, 6). In particular among all of these polymers, hydrogels allow for preparation of modified drug delivery systems, both in terms of time and in terms of targeting (7, 8). Hydrogels are crosslinked polymeric structures that can absorb great quantities of water or biological fluids. Due to the nature of their hydrophilic chains, hydrogels are thermodynamically compatible with water. This feature allows them to swell in aqueous media. Hydrogels have many applications in the biomedical and pharmaceutical fields because, as a result of their properties, they are very similar to living tissue compared to other synthetic materials. For these reasons they have been used in contact lenses, membranes for biosensors, and as materials for the construction of artificial skin and devices for releasing drugs and proteins (9, 10).

Ellagic acid is a depside, present in dried fruits and berries, including raspberries, strawberries, blackberries, cranberries, pomegranates and walnuts, with different EAHling properties such as anti-oxidant, anti-bacterial, anticancer and anti-viral (11-14). Numerous studies have shown that ellagic acid has anticancer activity towards tumor cells of breast, esophagus, pancreas, skin and prostate (15-17). The mechanism of action of ellagic acid on tumor cells consists in the inhibition and in the blocking of their replication and in the induction of their apoptosis (18).

The purpose of this work was to design, prepare and characterize a potentially useful prodrug for breast cancer treatment. In particular, a L-glycine and ellagic acid-based derivative was functionalized with a polymerizable group, polymerized and loaded with folic acid (FA) (Figure 1).

The function of FA in this case is to selectively and actively target the attachment of the implant towards tumor cells, which are known to overexpress FA receptors on their surface (19-21). To achieve this goal, L-glycine and ellagic acid, were functionalized with a polymerizable group and loaded with FA to make it more natural, non-toxic, compatible and specific for the site of action. The obtained compounds were characterized by Fourier Transform Infrared (FT-IR) and Proton Nuclear Magnetic Resonance (^{1}H-NMR) spectrometries. The ability of the obtained hydrogel to protect against lipid peroxidation induced by *tert*-BOOH, was examined in rat liver microsomal membranes. Cytotoxicity towards breast cancer cells was also evaluated. The data showed a significant decrease in viability of cells exposed to hydrogel ellagic acid-based containing folic acid.

Correspondence to: Roberta Cassano, Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Edificio Polifunzionale, Via Pietro Bucci, 87036 Arcavacata di Rende, Cosenza, Italy. Tel/Fax: +39 984493227, e-mail: roberta.cassano@unical.it

Key Words: Hydrogel, ellagic acid, glycine, folic acid, anti-oxidant, breast cancer, implant.

The results of all studies indicated the possibility that the ellagic acid-based hydrogel may find use, as subcutaneous implant, in the treatment of tumors whose conventional therapy provides the medication causing the onset of serious side-effects.

Materials and Methods

Reagents. All solvents of analytical grade were purchased from Carlo Erba Reagents (Milan, Italy): acetone, chloroform, dichloromethane, ethanol, ethyl ether, methanol, *n*-hexane, acetonitrile and triethylamine. L-Glycine (MW=75.07), 4,4'-dimetoxytrityl chloride (DMTrCl), trimethyl chlorosilane (Me_3SiCl), ellagic acid (MW=302.19), dicyclohexyl carbodiimide (DCC), dimethylaminoyiridine (DMAP), acryloyl chloride, ammonium persulfate $(NH4)_2S_2O_8$, *N*,*N*-dimethylacrylamide (DMAA), FA, *tert*-butyl hydroperoxide (t-BOOH) trichloroacetic acid (TCA) acid, 2-thiobarbituric (TBA), butylated hydroxytoluene (BHT), 4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO, USA).

Cell culture. MCF-7 and MDA-MB-231 cells, obtained from the American Type Culture Collection (Manassas, VA, USA), were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 containing 5% fetal bovine serum and supplemented with 1% L-glutamine and 1% penicillin/streptomycin (Sigma, Milan, Italy). The cells were cultured in serum-free medium (SFM) for at least 24 h before treatments.

Instruments. Infrared spectra were performed on KBr pellets using an FT-IR Perkin-Elmer 1720 spectometer (Norwalk, Connecticut, USA), in the range 4000-400 cm^{-1} (16 scans). The ^{1}H-MNR were performed by a Bruker VM30 spectometer (Bruker, Zürich, Switzerland), the chemical shifts are expressed in δ and are related to the solvent. The structures of synthesized compounds was confirmed by (GC-MS) Hewlett Packard 5972 (HAZLET, New Jersey, USA). The UV-VIS spectra were carried out using JASCO-530 UV spectrophotometer (JASCO Europe s.r.l., Milan, Italy). Samples were freeze-dried using a freeze-drying Micro Modulyo Edwards apparatus (Hastings, UK).

Synthesis of N-trytilglycine (1). The reaction was conducted according to the procedure reported in literature (22). In a three-neck flask fitted with a reflux condenser, funnel dripper, magnetic stirrer, thoroughly flamed and maintained under nitrogen bubbling, L-glycine (0.27 g, 13 mmoles) was dissolved in 1.22 ml mixture of chloroform (0.98 ml) and acetonitrile (0.24 ml). The reaction mixture was maintained at 30˚C under magnetic stirring. After dissolution, 0.42 ml (3.3 mmol) of trimethyl chlorosylane were added. The reaction was conducted for 2 h at 30˚C. Thereafter, 0.97 ml (6.9 mmol) of dry triethylamine and dropwise 4,4'-dimethoxytrytil chloride (1.20 g, 13 mmol), dissolved in chloroform (15.77 ml) were added. The addition of 4,4'-dimethoxytrytil chloride caused a chromatic change from white to red. This mixture was maintained for one hour under magnetic stirring; then 0,71 ml of methanol were added causing chromatic change from red to grey-green. The mixture was left stirring for 2 h. Finally the colour was pale yellow. The reaction was monitored through (TLC) on aluminum oxide (eluent mixture: chloroform). The solvents were then evaporated under reduced pressure and the obtained solid was washed with diethyl ether and then with an aqueous solution of citric acid 5% w/v. The organic phase was treated with a solution of NaOH 1 N (7.18 ml) and water (3.59 ml). The phase was treated with cold diethyl ether (17.18 ml) and neutralized with glacial acetic acid and the obtained precipitate was extracted with diethyl ether, while the organic phase was washed with distilled water and dried for 2 h with magnesium sulphate. The solution was filtered and dried under reduced pressure. The obtained product (**1**), orange in colour, was characterized through FT-IR.

Esterification of N-trytilglycine with ellagic acid (2). The reaction was conducted in agreement with the procedure reported in literature (23). In a two-neck flask fitted with a reflux condenser, magnetic stirrer, thoroughly flamed and maintained under nitrogen bubbling, 0.50 g (1.32 mmol) of (**1**) and 0.39 g (1.32 mmol) of ellagic acid were dissolved in 2.64 ml of dry dichloromethane. After dissolution, DCC (0.35 g, 1.7 mmol) and DMAP (0.08 g, 0.65 mmol) were added. The reaction mixture was maintained at room temperature under magnetic stirring for 12 hours. The reaction was monitored through TLC on silica gel (eluent mixture chloroform/n-exane 5:5). Finally, the product was filtered to be purified from dicyclohexyl urea formed during the reaction. After that it was purified through a chromatographic column on silica gel (eluent mixture dichloromethane/petroleum ether 4:6). The obtained product (**2**) was characterized through FT-IR.

Detritylation reaction (3). The reaction was conducted in agreement with the procedure reported in literature (24). In a three-neck flask fitted with a reflux condenser, magnetic stirrer, thoroughly flamed and maintained under nitrogen bubbling, 0.15 g of (**2**) (0.22 mmol) were added to a solution of trifluoroacetic acid in dichloromethane 60% giving a red-colored solution. After that, 0.45 ml of methanol (0.59 mmol) were added and the mixture was stirred for one hour at room temperature until the observation of a chromatic shift from red to yellow-orange. The solvent was evaporated at reduced pressure and the obtained salt was dried. This latter was subsequently washed with 2.26 ml of a 1 N HCl solution in MeOH. The solvent was evaporised and the product was dried and washed with diethyl ether in order to eliminate the trytil moieties. The obtained product (3) was dried under reduced pressure and characterized through FT-IR and ^{1}H-NMR.

Acrylation of 3. In a three-neck flask fitted with a reflux condenser, magnetic stirrer, thoroughly flamed and maintained under nitrogen bubbling, product **3** (0.30 g, 0.83 mmol) was dissolved in 6 ml of dichloromethane. After that, 0.08 ml (0.99 mmol) of acryloil chloride and 0.14 ml (0.1 mmol) of triethylamine were added and the solution appeared yellow coloured. The reaction was maintained for 12 hours at room temperature and was monitored through TLC on silica gel (eluent mixture chloroform/methanol 9:1). Finally, the solvent was evaporated under reduced pressure and the obtained product (**4**) was characterized by FT-IR e ^{1}H-NMR.

Hydrogel preparation. In a two-neck flask fitted with a reflux condenser, magnetic stirring thoroughly flamed and maintained under nitrogen bubbling, product **4** (0.65 g, 1.57 mmol) was solubilized in an aqueous solution of NH_3/urea. Subsequently, DMAA (0.16 ml, 0.1 mmol) and ammonium persulphate (80.35 g, 0.35 mol) were added. Finally, the reaction was carried out at 60˚C

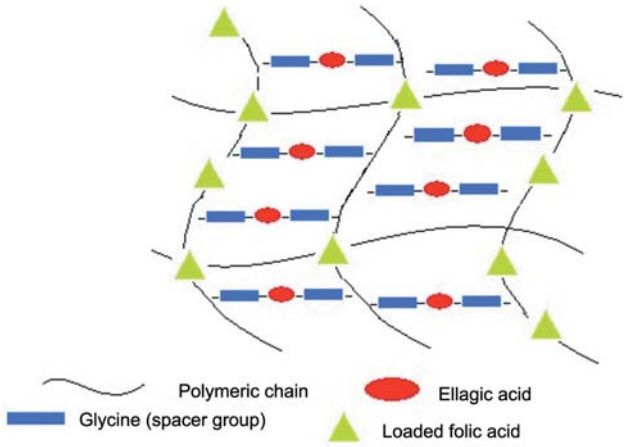

Figure 1. *Ellagic acid and glicine-based hydrogel containing folic acid.*

until the formation of the hydrogel. The obtained hydrogel was washed with diethyl ether in a porous filter, dried under vacuum and characterized through FT-IR (9).

Antioxidant activity evaluation. The ability of the obtained hydrogel to protect against lipid peroxidation induced by *tert*-BOOH, was examined in rat liver microsomal membranes during 120 min of incubation. Aliquots of both ellagic acid and ellagic acid-based hydrogel (EAH) were added to the microsomal suspension (9, 25-27). Then the suspensions were incubated at 37˚C in a shaking bath under air in the dark. After incubation, thiobarbituric acid-malondialdehyde complex (TBA-MDA) formation was monitored by the use of UV-VIS spectrophotometry at 535 nm (25). The experiment was repeated in triplicate (n=3).

Swelling studies. The swelling characteristics of the hydrogel were determined in order to check for its hydrophilic affinity (9). Typically, aliquots (40-50 mg) of hydrogel dried to constant weight were placed on a tared 5-ml sintered glass filter (Ø10 mm; porosity, G3), weighed, and left to swell by immersing the filter plus support in a beaker containing the swelling media, namely phosphate buffers at pH 6.2 to mimic the conditions typical of tumor pathology, and pH 7.4 to simulate the physiological environment. At a predetermined time, the excess of water was removed by percolation at atmospheric pressure. Then the filter was placed in a centrifuge test tube, fixed with the help of a bored silicone stopper, then centrifuged at 1372 rcf for 15 min and weighed. This operation was repeated at the different times (1, 6, 12 and 24 h). The filter weight was determined after centrifugation with water alone. The weights recorded at the different times were averaged and used to give the water content (α, %) by the following equation:

$$\alpha\% = \frac{(Ws - Wd)}{Wd} \times 100$$

where Ws and Wd are weights of swollen and dried hydrogel, respectively.

Incorporation of FA into preformed hydrogel. The folic acid incorporation into microspheres was performed (9) as follows: 500 mg of preformed empty hydrogel (prepared as described above) were wetted with 6 ml in a concentrated solution of folic acid (15 mg/ml). After three days, under slow stirring at 37˚C, the microspheres were filtered and dried at reduced pressure in the presence of P_2O_5 to a constant weight. The loading efficiency (LE, %) of all samples were determined by UV-Vis spectrophotometric analysis of filtered solvent according to the following equation:

$$LE(\%) = Ci - C0/Ci \times 100$$

where C_i was the concentration of FA in solution before the loading study, C_0 the concentration of FA in solution after the loading study. LE was measured spectrophotometrically (λ=365 nm, ε=7595 $mol^{-1}dm^3cm^{-1}$).

Drug release studies. Dried hydrogel (10 mg) was dispersed in 6 ml of swelling media (phosphate buffers at pH 6.2 and pH 7.4 (9). The test tubes were maintained at 37˚C in an horizontal shaking bath and shaked at a rate of 100 rpm. At predetermined intervals, the samples were centrifuged, 5 ml of supernatant were removed and the medium was replaced with fresh solution to maintain the same total volume throughout the study. The concentration of FA was determined spectrophotometrically at 365 nm. The experiment was repeated in triplicate (n=3). The data were calculated in terms of the drug release percentage.

(MTT) assay. The cytotoxic effects of EAH and of EAH containing folic acid on human breast cancer cells were evaluated by MTT dye test (cell viability) (27, 28). The cells were seeded at a density of 1×10^4 cells in 96-well tissue culture plates for 24 h at 37˚C and 5% CO_2 to allow the adhesion of the cells. After 24 hours of incubation, the culture medium was replaced with SFM and EAH or EAH+FA were added at 50 μM. The toxicity experiments were carried out at 24 and 48 h of incubation. Untreated cells were used as control.

At the end of the incubation time, 50 μl of MTT tetrazolium salt (5 mg/ml dissolved in SFM) were added to each well. The plates were incubated for an additional four hours and then the medium was discarded. A volume of 150 μl of DMSO was added to each well, and the solution was vigorously mixed to dissolve the reacted dye. The absorbance of each well was read on a microplate reader (Multiskan EX, Thermo Scientific, USA) at a test wavelength of 570 nm with a reference wavelength of 690 nm. The sample was tested in triplicate and the percentage of viable cells, directly proportional to the amount of formazan crystals formed, was calculated using the following equation: % Cell viability=AbsT/AbsU ×100, where AbsT is the absorbance of treated cells and AbsU is the absorbance of untreated cells.

Statistical analysis. Data are expressed as the mean±S.D. of at least three independent experiments. Statistical analysis was performed using Student's *t*-test. *p*-Values ≤0.05 were considered statistically significant.

Results and Discussion

In recent years, the interest in more greatly biocompatible materials, useful for the controlled and site-specific release of active molecules, has been increasing. In this context the aim of this work was the preparation of a hydrogel, made of ellagic acid and L-glycine, to be used as a subcutaneous implant.

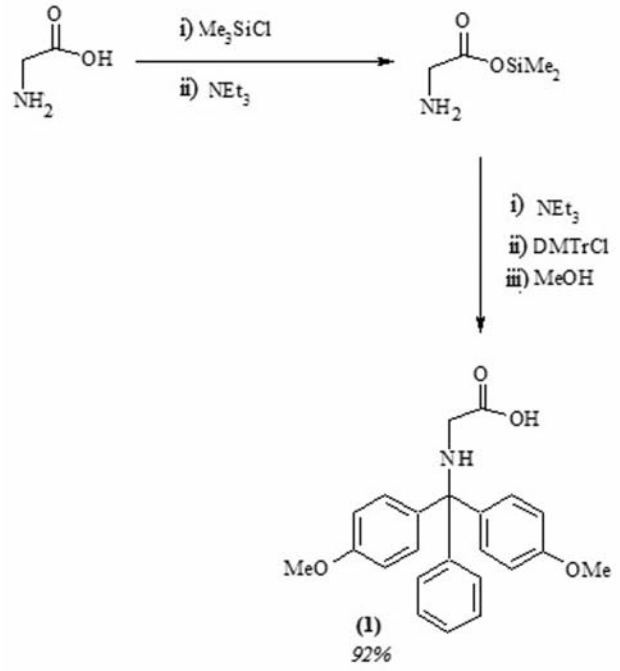

Figure 2. *Synthetic route to N-trytil glycine.*

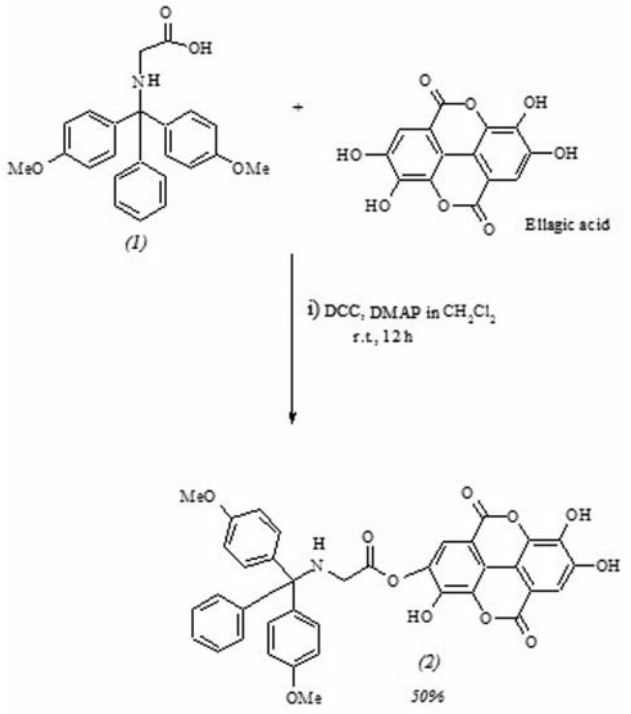

Figure 3. *Esterification reaction.*

L-Glycine is an amino acid that, having two sites of functionalization, can act as a spacer group between an active substance, such as ellagic acid, and a polymerizable group such as acrylic acid. In fact, thanks to the presence of an acrylic moiety, it is possible to perform co-polymerization reactions to obtain a hydrogel potentially employable as subcutaneous implant in the treatment of breast cancer.

The first step of the whole process was the protection of the aminic group of glycine conducted in the presence of trimethyl chlorosilane, a temporary protecting group for the carboxylic function. For this purpose, the reaction was carried out using triethylamine and methanol to enhance the binding of trimethyl chlorosilane and its elimination after protection of the aminic group. DMTrCl was chosen as protecting group of the aminic moiety since it is very effective but easily removable one. Both the protections, the temporary one for –COOH and the stable one for -NH_2, were used in stoichiometric amounts. The most likely mechanism of action involves the binding of the carboxyl group of glycine with Me^3SiCl, releasing a mole of hydrochloric acid. After that DMTrCl was added and it was attacked on its electrophilic carbon by the nucleophilic -NH_2 releasing another mole of hydrochloric acid. The addition of methanol resulted in the liberation of Me_3SiCl from the carboxyl group of glycine (Figure 2). The product of this first step was characterized by the most common spectroscopic techniques: FT-IR (KBr) ν (cm^{-1}): 3066 e 3035 (aromatics -CH), 1725 (-COOH), 1654 (-CONH). M/Z: 320 (63 %), 77 (38 %). ^{1}H-NMR ($CDCl_3$) δ (ppm): 9.80 (s, 1H), 6.90-7.94 (m, 13H), 4.04 (s, 2H), 3.85 (s, 6H) 3.61 (sb, 1H). Yield: 92%.

The second step of synthesis was the esterification of the carboxyl group of glycine with one of the hydroxyl groups of ellagic acid. For this condensation, a coupling agent such as DCC that binds the -OH group of -COOH makes it become more electrophilic towards nucleophilic attack of hydroxyl groups of ellagic acid. A base, DMAP, was used as buffer system and nucleophilic catalyst to promote the deprotonation of the hydroxyl groups of ellagic acid that become more nucleopilic. The esterification involved the formation of dicyclohexyl urea (DCU) as a leaving group that can be removed by filtration (Figure 3). The obtained product (**2**) was dried under vacuum, purified through a chromatographic column and characterized through FT-IR and ^{1}H-NMR: FT-IR (KBr) ν (cm^{-1}): 3068 e 3034 (aromatics -CH), 1769 (-C=O ester), 1718 (-C=O ester), 1653 (-CONH). ^{1}H-NMR ($CDCl_3$) δ (ppm): 6.80-7.88 (m, 15H), 3.91 (s, 2H), 3.82 (s, 6H). Yield: 50%.

The third step involved the removal of trityl group linked to the amino group of glycine in order to make it free for the next step of acrylation. This protecting group was removed by using a solution of trifluoroacetic acid 60% v/v in dichloromethane. This reaction gives the corresponding

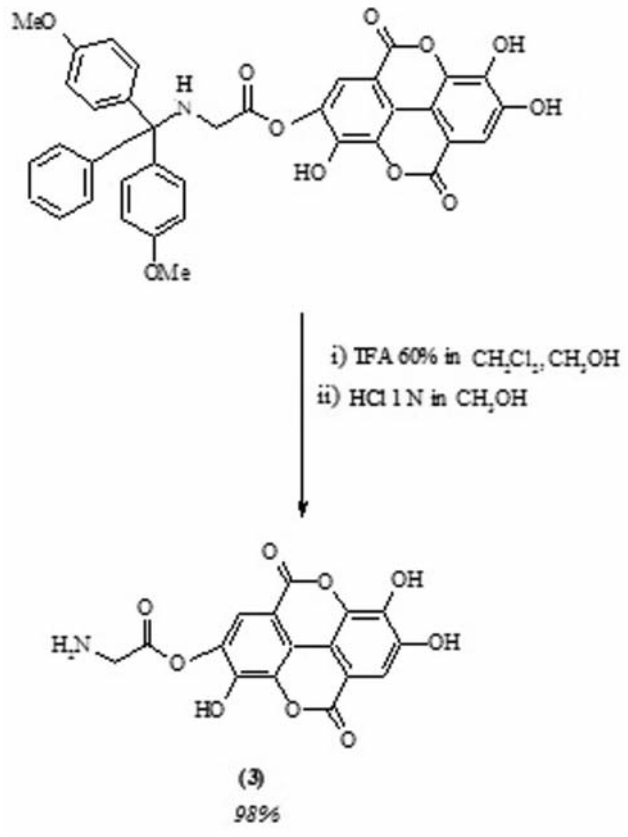

Figure 4. *Deprotection*.

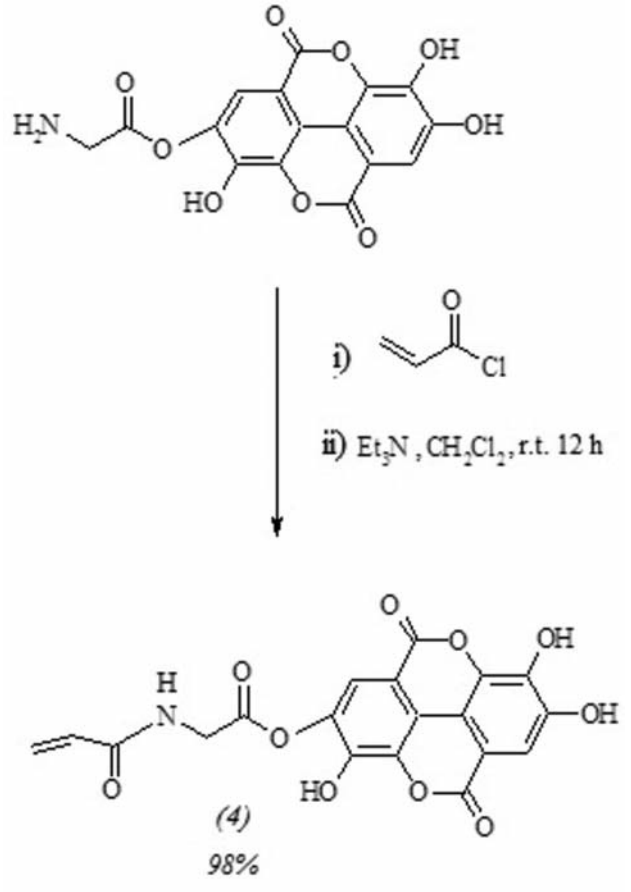

Figure 5. *Acrylation*.

chlorhydrate amine (-NH_2 ×HCl); to obtain the free aminic group, it was necessary to add a solution of 1 N hydrochloric acid in methanol to the dry salt. In order to remove all the free protecting groups the product (**3**) was washed with diethyl ether dried under vacuum and characterized through FT-IR and ^{1}H-NMR (Figure 4). FT-IR shows the disappearance of the amidic band at 1653 cm^{-1} and the preservation of characteristic stretching of ester bonds: ^{1}H-NMR (C_2D_6SO) δ (ppm): 7.25 (s, 1H), 7.10 (s, 1H), 4.04 (s, 2H). Yield: 98%.

The hydroxyl groups of ellagic acid linked to glycine were esterified using acryloyl chloride. The mechanism of this reaction consisted in the nucleophilic attack of the amino group of L-glycine towards the electrophilic carbonyl of acryloil chloride by liberation of HCl (Figure 5). The insertion of an acrylic moiety was important for the step of co-polymerization. The obtained product (4) was characterized through FT-IR and ^{1}H-NMR: FT-IR (KBr) ν (cm^{-1}): 3067, 3034 (aromatics -CH), 1767 (-C=O ester), 1722 (-C=O ester), 1661 (-CONH), 995, 914 (vinyl group). 1H-NMR (C_2D_6SO) δ (ppm): 7.30 (s, 1H), 7.05 (s, 1H), 6.43 (dd, 1H), 6.11 (dd, 1H), 5.80 (dd, 1H), 4.10 (s, 2H). Yield: 98%.

The product (4) was dissolved in a NH_3/urea aqueous solution then ammonium persulfate and dimethyl acrylamide, a monofunctional co-monomer, were added at a temperature of 60˚C. The reaction was carried out until the formation of the hydrogel occurred and in the end the obtained product was washed several times with distilled water, dried under vacuum and characterized through FT-IR. This latter shows the disappearance of the bands attributable to the acrylic double bond.

The ability of the obtained hydrogel to inhibit lipid peroxidation in rat liver microsomal membranes during 120 minutes of incubation was examined and compared to the antioxidant activity of free ellagic acid. The results revealed (Figure 6) that the ability of the EAH to inhibit lipid peroxidation was time-dependent and follows a similar trend to that of ellagic acid.

The swelling behaviour of hydrogel were examined at two different pHs, 6.2, simulating the pathological cancerous environment, and 7.4 the effective physiological pH. The values were examined at different time-points (1, 6, 12 and 24 h). Each experiment was carried out in triplicate and the results

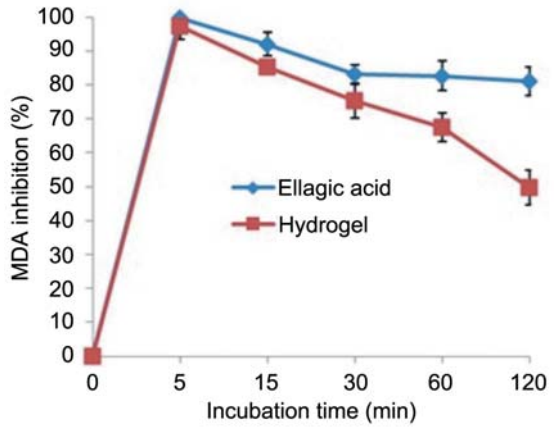

Figure 6. *Anti-oxidant activity evaluation. MDA: Malondialdehyde. Data are expressed as the mean±S.D. of at least three independent experiments.*

were in agreement within ±4% standard error. The swelling percent (α%) for the prepared material is reported in Table I.

The FA was entrapped into the preformed EAH by a soaking procedure consisting in the dissolution of FA in a solution that allows the swelling of polymeric matrix. Loading efficiency was calculated by UV-Vis spectrophotometric analysis of the filtered solution (λ=365 nm, =7595 $mol^{-1}dm^3cm^{-1}$). The experiment was repeated in triplicate and results showed that the drug was almost completely loaded into the matrix or on its surface. The entrapment efficiency was 75%±1.8.

The release studies of FA were conducted on aliquots of preformed hydrogel at two different pHs (6.2 and 7.4) and at different time-points (1, 6, 12 h and 24 h) by using a shaking water bath at 37˚C (body temperature). The results showed that FA was characterized by a slow kinetic-release, especially at pH 6.2 (Figure 7).

The cytotoxic effect of EAH on MCF-7 cells was evaluated. The results of this study demonstrated that the addition of EAH (50 μM) alone to MCF-7 cells for 24 and 48 h did not lead to statistically significant cytotoxic effects as compared with untreated cells (Figure 8). In addition, in order to evaluate the anti-tumoral effect of EAH+FA, an MTT assay was carried out in MCF-7 cells exposed to the polymeric system. As shown in Figure 8, a significant reduction (*$p<0.05$ *vs.* control) of viability was observed in the cells exposed for 24 and 48 h to 50 μM EAH+FA (Figure 8). Notably, EAH+FA dramatically increased anti-tumoral activity, inducing 53% cell death *vs.* control at 24 h, and actually reaching 62% suppression of viability after 48 h. Similar data were obtained in MDA-MB-231 cells (data not shown). These results suggest that the utilization of the polymeric system EAH+FA for localized breast cancer therapy may have a high potential for success.

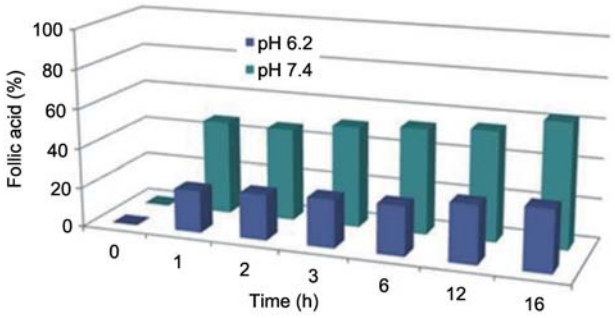

Figure 7. *In vitro release studies. Each experiment was carried out in triplicate and the results were in agreement within ±4% standard error.*

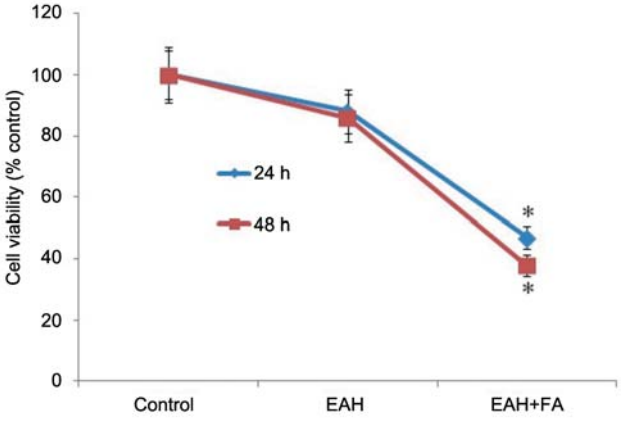

Figure 8. *Effect of formulation on MCF-7 cell viability. *$p<0.05$ compared to control. Data are expressed as the mean±S.D. of at least three independent experiments.*

Table I. *Swelling behavior of hydrogel.*

Time (h)	Swelling (α%)	
	pH 6.2	pH 7.4
1	125	482
6	224	712
12	252	752
24	277	771

α% was calculated as

$$\alpha\% = \frac{(Ws - Wd)}{Wd} \times 100$$

where Ws and Wd are weights of swollen and dried hydrogel, respectively.

Conclusion

The aim of the present work was the realization of a biocompatible hydrogel, based on ellagic acid and glycine, carrying FA, a model molecule, useful in subcutaneous implant in breast cancer treatment. The results confirmed the possibility that polymeric systems based on natural substances may find use in the treatment of cancer, where conventional therapy provides the onset of serious side-effects. The degree of swelling of the hydrogel not containing FA was studied at two different pH values and at fixed time intervals. In particular, we used phosphate buffers at pH 6.2 to mimic conditions typical of tumor pathology, and pH 7.4 to simulate the physiological environment. The obtained results showed that this hydrogel swelled more at pH 7.4. This could be attributed to the numerous phenolic groups, which being predominantly in their ionic form, increased the hydrophilicity of the ellagic acid hydrogel. This latter, loaded with FA, was also subjected to preliminary release studies conducted at pH 6.2 and pH 7.4. These studies have shown that FA was released similarly from the matrix irrespective of pH. More precisely, the release occurred slowly during the first 6 h and then increased gradually, probably due to the greater swelling of the hydrogel. In particular, FA was released over time in a relatively small quantity, therefore, it can be concluded that more drug could be released after 24 h. It is also interesting to note that FA was characterized by a slow kinetic release, especially at pH 6.2, typical of the cancerous environment. In addition, *in vitro* studies demonstrated that EAH containing FA, significantly inhibited the growth of human breast cancer cells. Our results show how EAH+FA exerted a rapid (as soon as after 24 h of treatment) and sustained cytotoxic effect, causing almost 62% of cell death within 48 h of exposure, if compared to EAH-alone, which was only able to exert a cytostatic effect. Finally, studies aimed at assessing antioxidant activity of the hydrogel not containing FA have shown the preservation of ellagic acid behavior. These studies were performed by monitoring the levels of malondialdehyde in rat liver microsomal membranes and revealed the ability of the hydrogel (EAH) to inhibit lipid peroxidation following the same trends of ellagic acid.

In conclusion, on the basis of the obtained results we can hypothesize a possible use of EAH as a subcutaneous implant for controlled and site-specific release of FA useful in the treatment of breast cancer.

Acknowledgements

This work was financially supported by MIUR (Programma di Ricerca di Rilevante Interesse Nazionale 2008) and University of Calabria funds. This work was also supported by Associazione Italiana Ricerca sul Cancro (AIRC) [MFAG grant number 6180 to Loredana Mauro and IG grant number 11595 to Sebastiano Andò] and by European Commission/FSE/Regione Calabria to Emilia Ricchio. The Authors are solely responsible for the work.

References

1 Cassano R, Trombino S, Ferrarelli T, Bilia AR, Bergonzi MC, Russo A, De Amicis F and Picci N: Preparation, characterization and *in vitro* activities evaluation of curcumin based microspheres for azathioprine oral delivery. React Funct Polym *72*: 446-450, 2012.

2 Trombino S, Cassano R, Ferrarelli T, Isacchi B, Bilia AR and Picci N: Collagen α-tocopherulate for topical applications: Preparation, characterization and antioxidant activity evaluation. Macromol Res *20*: 939-943, 2012.

3 Trombino S, Cassano R, Ferrarelli T, Cavalcanti P, Giraldi C, Lai F, Loy G and Picci N: Synthesis and *in vitro* antitubercular activity of isoniazid-gelatin conjugate. J Pharm Pharmacol *64*: 712-718, 2012.

4 Trombino S, Cassano R, Ferrarelli T, Mauro MV, Giraldi C, Manconi M, Fadda AM and Picci N: Respirable rifampicin-based microspheres containing isoniazid for tuberculosis treatment. J Biomed Mater Res Part A *100A*: 536, 2012.

5 Langer R: Biomaterials in drug delivery and tissue engineering: One laboratory's experience. Acc Chem Res *33*: 94-101, 2000.

6 Lu Y and Chen SC: Micro and nano-fabrication of biodegradable polymers for drug delivery. Adv Drug Deliv Rev *56*: 1621-1633, 2004.

7 Hoare T and Kohane DS: Hydrogels in drug delivery: Progress and challenges. Polymer *49*: 1993-2007, 2008.

8 Qiu Y and Park K: Environment-sensitive hydrogels for drug delivery. Adv Drug Deliv Rev *64*: 49-60, 2012.

9 Cassano R, Trombino S, Muzzalupo R, Tavano L and Picci N: A novel dextran hydrogel linking *trans*-ferulic acid for the stabilization and transdermal delivery of vitamin E. Eur J Pharm Biopharm *72*: 232-238, 2009.

10 Trombino S, Cassano R, Bloise E, Muzzalupo R, Tavano L and Picci N: Synthesis and antioxidant activity evaluation of a novel cellulose hydrogel containing trans-ferulic acid. Carbohydr Polymer *75*: 184-188, 2009.

11 Talcott ST and Joon-Hee L: Ellagic acid and flavonoid antioxidant content of muscadine wine and juice. J Agric Food Chem *50*: 3186-3192, 2002.

12 Seeram NP, Adams LS, Henning SM, Niu Y, Zhang Y, Nair MG and Heber D: *In vitro* antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. J Nut Biochem *16*: 360-367, 2005.

13 Festa F, Aglitti T, Duranti G, Ricordi R, Perticone P and Cozzi R: Strong antioxidant activity of ellagic acid in mammalian cells *in vitro* revealed by the comet assay. Anticancer Res *21*: 3903-3908, 2001.

14 Yu YM, Chang WC, Wu CH and Chiang SY: Reduction of oxidative stress and apoptosis in hyperlipidemic rabbits by ellagic acid. J Nutr Biochem *16*: 675-681, 2005.

15 Stoner GD and Gupta A: Etiology and chemoprevention of esophageal squamous cell carcinoma. Carcinogenesis *22*: 1737-1746, 2001.

16 Larrosa M, Tomas-Barberan FA and Espin JC: The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway. J Nutr Biochem *17*: 611-625, 2006.

17 Dorai T and Aggarwal BB: Role of chemopreventive agents in cancer therapy. Cancer Lett *215*: 129-140, 2004.
18 Han DH, Lee MJ and Jeong HK: Antioxidant and apoptosis-inducing activities of ellagic acid. Anticancer Res *26*: 3601-3606, 2006.
19 Lucock M: Folic acid: Nutritional biochemistry, molecular biology, and role in disease processes. Mol Gen Metab *71*: 121-138, 2000.
20 John F, Ross BS, Prabir K, Chaudhuri MD and Ratnam M: Differential regulation of folate receptor isoforms in normal and malignant tissues *in vivo* and in established cell lines. Physiologic and clinical implications. Cancer *73*: 2432-2443, 1994.
21 Guo M, Que C, Wang C, Liu X, Yan H and Liu K: Multifunctional superparamagnetic nanocarriers with folate-mediated and pH-responsive targeting properties for anticancer drug delivery. Biomaterials *32*: 185-194, 2011.
22 Barlos K, Papaioannou D and Theodoropoulos D: Efficient "one-pot" synthesis of *N*-trityl amino acids. J Org Chem *47*: 1324-1326, 1982.
23 Vanhaecht B, Teerenstra MN, Suwier DR and Koning CE: Dicyclohexylcarbodiimide assisted synthesis of aliphatic polyesters at room temperature. J Macromol Sci Part A: Pure Appl Chem *37*: 633-643, 2000.
24 Theodorou V, Ragoussis V, Strongilos A, Zelepos E, Eleftheriou A and Dinitriou M: A convenient method for the preparation of primary amines using tritylamine. Tetrahedron Lett *46*: 1357-1360, 2005.
25 Trombino S, Cassano R, Bloise E, Muzzalupo R, Leta S, Puoci F and Picci N: Design and synthesis of cellulose derivatives with antioxidant activity. Macromol Biosci *8*: 86-95, 2008.
26 Trombino S, Serini S, Di Nicuolo F, Celleno L, Andò S, Picci N, Calviello G and Palozza P: Antioxidant effect of ferulic acid in isolated membranes and intact cells: synergistic interactions with alpha-tocopherol, beta-carotene, and ascorbic acid. J Agric Food Chem *52*: 2411-2420, 2004.
27 Trombino S, Cassano R, Muzzalupo R, Pingitore A, Cione E and Picci N: Stearyl ferulate-based solid lipid nanoparticles for the encapsulation and stabilization of beta-carotene and alpha-tocopherol. Coll Surf B: Biointer *72*: 181-187, 2009.
28 Panno ML, Giordano F, Rizza P, Pellegrino M, Zito D, Giordano C, Mauro L, Catalano S, Aquila S, Sisci D, De Amicis F, Vivacqua A, Fuqua FW and Andò S: Bergapten induces ER depletion in breast cancer cells through SMAD4-mediated ubiquitination. Breast Cancer Res Treat *136*: 443-455, 2012.

Received August 5, 2013
Revised September 27, 2013
Accepted September 30, 2013