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ARCT'ALG® IMMOBILIZATION AND RELEASE FROM HYDROGEL MEMBRANES

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Abstract. *The hydrogel properties make it attractive for a variety of biomedical and pharmaceutical applications, primarily in drug delivery system. In view of the large advanced innovations in cosmetic products by the introduction of new active agents as the matrices used for its controlled release, the objective of this work was to evaluate the release of a natural active agent, the Arct'Alg® immobilized in hydrogel membranes. Study on physical-chemical properties and in vitro biocompatibility of poly(vinyl-2- pyrrolidone) (PVP) and poly(vinyl alcohol) (PVA) hydrogel membranes obtained by ionizing radiation crosslinking had been performed. The physical-chemical characterization of these polymeric matrices was carried out by gel fraction and swelling tests and biocompatibility study by in vitro test of cytotoxicity using the neutral red uptake technique. The hydrogel membranes prepared with Arct'Alg® incorporation were submitted to the release test in phosphate buffer solution pH 5.0 in a stirrer incubator at 37°C and the released active agent was quantified by high performance liquid chromatography (HPLC), also the released Arct'Alg® was submitted to an in vitro cytostimulation test on cellular culture. The results obtained showed a high crosslinking degree in both hydrogels, a PVP greater swelling percentage in relation to PVA hydrogel, no cytotoxic effect in the cytotoxicity assay and in the kinetics of release the PVP hydrogel membranes showed Arct'Alg® release rate about 20% higher than PVA hydrogel membranes. In the cytostimulation test the PVP device showed cell population increase of about 80%. These results showed that Arct'Alg® immobilized in PVP hydrogel membrane could be used in skin repair process due to cytostimulation property, in the cosmetic application.*

Keywords: *Hydrogel, Delivery System, Cytotoxicity, Cytostimulation*

1. INTRODUCTION

Cosmetic industries due to their great market size is promoting technological run to innovate and improve their products by adding new active agents or introducing hydrogel for controlled release dressings to the market.

Hydrogel matrix, which is a three-dimensional structure, is generally made up of hydrophilic polymers like poly(vinyl alcohol), poly(vinyl pyrrolidone), poly(acrylic acid), etc. and holds significant amount of water in its porous structure. (Varshney, 2007).

Hydrogels were studied by researchers long time before the polymers existence and recognized use. Wichterle and Lim proposed their use as contact lenses in 1960 and this opened the door to other biomedical applications for hydrogels, including drug delivery (Gehrke and Lee, 1990).

Controlled drug-delivery systems are designed to deliver the drugs at desirable times and/or specific sites to achieve the therapeutic objective. Hydrogels are one of the upcoming classes of polymer-based controlled release drug delivery systems (Chaoling, Kim and Lim, 2008; Gupta and Vermani, 2002).

Arct'Alg® is a standardized extract derived from red algae, the *Chondrus crispus*, which grows in cold waters, as Arctic Sea. Arct'Alg® has in its composition the dipeptide citrullyl-arginine which has biological properties as antioxidant, lipolytic, anti-inflammatory and cytostimulation action (Exsymol, 2005).

The Arct'Alg® cytostimulation property has a great importance in skin repair processes. This active agent in contact with the fibroblastic cells promotes a differentiation and proliferation of them occurring synthesis of collagen and elastic fibers, very important to tissue regeneration. Collagen synthesis is a complex biochemical process which is still not fully known. The fibroblasts present in the injured tissue during the regeneration process are characterized by a high production of collagen and spontaneously synthesize nitric oxide (NO). From arginine, by a reaction mediated by nitric oxide synthase, the NO is produced in inflammatory conditions and is an important mediator in several biological processes or via arginase to ornithine and urea (Witte et al., 2002).

Arginine, a constituent of Arct'Alg®, is a semi essential amino acid and becomes to essential in a stress situation such as trauma, skin repair and wound healing (Witte et al., 2002). In tissue repair process, arginine is directly involved in cytostimulation, especially in skin damage.

The ornithine is a precursor of polyamines and proline. The polyamines are essential for cellular cytostimulation due to involvement in almost all stages of of DNA, RNA and proteins synthesis. Proline is essential for collagen fibers formation due to be collagen type III precursor (Giménez et. al., 2005).

In view of the large advanced innovations in cosmetic products by the introduction of new active agents on new matrix for obtaining better release system, the objective of this work was the incorporation of Arct'Alg® in PVP and PVA hydrogel membranes, study of its release kinetic and *in vitro* citrullyl-arginine cytostimulation action in skin cell, aiming the application in cosmetic area, for skin repair process.

2. MATERIALS AND METHODS

Two formulations were studied in order to verify the better hydrogel matrix to compose a delivery system of Arct'Alg®.

2.1 Preparation of hydrogel matrices

The synthesized hydrogel matrices were of PVP and PVA.

The PVP matrices were obtained from 6% of Poly (N-vinyl-pyrrolidone) (PVP) K 90, Kollidon® 90F, average molar weight from 1000000 to 1500000 from BASF, in aqueous solutions, using 1.5% of poly (ethylene glycol) (PEG 300) from Oxiteno as plasticizer and 0.5% of agar technical type 3 from Oxoid as gelling agent. The PVP hydrogel was obtained by mixed of PVP K90, PEG 300 and water. After 24 hours at room temperature, this solution was heated and added to the agar keeping in heat until complete dissolution of the components.

The PVA matrices were prepared with 8% of poly (vinyl alcohol) (PVA), Celvol® E47/88, degree of hydrolysis 87-89%, melting point 180ºC, glass transition temperature 58ºC, from Dermet Agekem, in aqueous solutions and 1% of agar technical type 3 from Oxoid as gelling agent. The PVA hydrogel was synthesized from the mixture of PVA and water. The PVA water solution was heated for complete dissolution and the agar was added under heat until dissolution of the components.

The membranes were prepared pouring 5mL of the hydrogel solution after cooling at approximately 40ºC, in circular molds, which were sealed, packed and irradiated in a gamma rays source of ^{60}Co, with 5.72 kGY/h^{-1} dose rate. The PVP matrices were irradiated at a dose of 25 kGy and the PVA at a dose of 20 kGy.

Samples of hydrogel matrices were characterized by gel fraction and swelling tests and the first biocompatibility test was verified by *in vitro* cytotoxicity assay.

2.2 Characterization of hydrogel matrices

Gel fraction. The soluble fraction removal of dried PVP and PVA hydrogels samples was performed in Soxhlet extractor using water as solvent during 36 hours. After this time the samples were dried until constant weight and the gel fraction was calculated as per Eq. (1) according to ASTM D 2765 (ASTM, 2001).

$$GF\ \% = w_f / w_i \times 100 \quad (1)$$

Where: w_i = initial dry weight
w_f = final dry weight

Swelling test. Dried PVP and PVA hydrogel samples were immersed in 20ml of phosphate buffered solution (PBS) pH 5.0 for a period of 24 hours. The hydrogel mass was checked every hour in the first 6 hours of the test, and after 24 hours. The swelling degree was calculated using Eq. (2) according to ASTM D 570 (ASTM, 1998).

$$S\ \% = (w_t - w_i)/w_i \times 100 \quad (2)$$

Where: w_i = initial weight
w_t = weight at each time

Cytotoxicity test. The *in vitro* cytotoxicity test was performed using the neutral red uptake technique, according to International Standard Organization (ISO 10993-5, 1992) and methodology previously published (Rogero *et al.,* 2003). Serially diluted extract of hydrogel samples, negative control and positive control were placed in contact with cells in a 96 wells flat-bottomed microplate. The used negative control was no toxic polyvinyl chloride (PVC) pellets and 0.02 % phenol solution as positive control and the used cell line was from mouse connective tissue, NCTC clone 929 (American Type Culture Collection). The cytotoxic effect was quantitatively assessed by measuring the neutral red incorporated by the viable cells, after rupture of them in the end of the assay, in spectrophotometer Sunrise - Tecan, in 540nm. The percentage of cell viability was calculated in relation to control cell and the toxic potential was determined by the cytotoxicity index ($IC_{50\%}$). The $IC_{50\%}$ is the concentration of extract which causes damage or death in 50% of cell population in the test.

2.3 Preparation of hydrogel devices

3% of Arct'Alg® was incorporated in each of the hydrogel solutions of PVP and PVA prepared in accordance with the methodology described in 2.1, after cooling at approximately 40ºC, mixed until complete homogenization. The hydrogel membranes devices were prepared pouring 5mL of the hydrogel solution containing the active principle in circular moulds which were sealed packed and irradiated with gamma rays of 5.72 kGY/h^{-}1 dose rate of Co-60 source (GammaCell 200, Atomic Energy of Canada Ltd.). The irradiation dose was 25 kGy for PVP device and 20 kGy for PVA.

2.4 Characterization of hydrogel devices

Obtained PVP and PVA hydrogel devices were submitted to an *in vitro* assay of Arct'Alg® release and the released active agent tested for it cytostimulation property.

In vitro kinetic release and quantification of Arct'Alg®. The Arct'Alg® kinetic release assay was carried out utilizing 2.5g of PVP or PVA hydrogel membrane device immersed in 35mL of PBS 0.1M pH 5.0 in a glass capped bottles, in triplicate, in an incubator under 120 rpm agitation at 37°C during 24 hours. Aliquots of 1mL were collected every hour in the first 7 hours of the test, and then, after 24 hours. In this test, in the same conditions, it was used PVA hydrogel membrane device containing citrullyl-arginine with the purpose to compare the chromatograms of citrullyl-arginine released with that one from Arct'Alg® devices.

The quantification of released Arct'Alg® were determined by HPLC equipment from Shimadzu, equipped with a binary pump and controlled with the CLASS-LC10 software. A total of 100µL of samples were injected into a reversed-phase C18 column (250mm x 4.6mm) from Varian, flow rate of 1 mL/min and UV detection at 200nm. The mobile phase consisted of 3% acetonitrile and PIC B6 97% from Waters in an isocratic system. The used standard was citrullyl-arginine. The peak area was obtained in the chromatogram by injection of 5µL of standard solution (0.2 mg citrullyl-arginine/mL). The released amount of citrullyl-arginine contained in Arct'Alg® was calculated by comparing its peak area with standard solution.

Cytostimulation test

The cytostimulation activity of Arct'Alg® from PVP and PVA hydrogel devices on skin cell culture was verified *in vitro* by of neutral red incorporation technique according to methodology in the literature (Christophe, 2006).

In sterile conditions, the PVP and PVA Arct'Alg® devices were placed in glass capped bottles with minimum Eagle´s medium (MEM) supplemented with Leibovitz n°15 (L-15) plus 2% fetal calf serum (FCS), one membrane per 24 mL. These bottles were placed in an incubator from Tecnal, model TE-420, for 1 hour under 120 rpm agitation at 37ºC to release the active agent. The final solutions containing released Arct'Alg® were named as PVP extract and PVA extract, respectively.

In this test four 96 wells flat bottomed microplates contained a cell line FPC – IAL of rabbit skin fibroblasts cells, seeded 200 µL of 10^5 cells/mL suspension. FPC – IAL cells were cultured in MEM and L-15 (v / v) plus 2% FCS. After obtained confluence, the medium of these microplates was replaced in the following conditions:

- Microplate 1 (cell control) - replaced by a deprived medium (deficit nutritional condition): MEM and L-15 (v / v) plus 2% FCS;
- Microplate 2 – replaced by a optimal medium (total nutritional condition): MEM and L-15 (v / v) plus 10% FCS;
- Microplate 3 – replaced by a PVP extract;
- Microplate 4 – replaced by a PVA extract.

The microplates were incubated at 37ºC for 48 hours in a humidified 5% CO_2 atmosphere. After this period the media were replaced by 200 µL of neutral red solution and incubated again for 3h. Then the neutral red solution was discarded and microplates washed with phosphate buffer solution pH 7.5 and subsequently with washing solution (1% of CaCl2 10% in formaldehyde 0.5%). After washing, the microplates wells were filled with 200 µL of extraction solution (50% of acetic acid 2% and 50% ethanol) for living cells lyses and release of neutral red and the optical density (OD) reading in spectrophotometer at 540 nm. With the

OD results was calculated the cell viability percentage in relation to control cell (microplate 1 considered 100%).

3. RESULTS AND DISCUSSION

The criterion for considering the hydrogel matrix appropriate or not, to be submitted to characterization tests, was the formation of a homogeneous film, transparent and elastic, these parameters evaluated visually and manually. PVP and PVA matrices formed a homogeneous film, transparent, with good adhesiveness and showed adequate strength to handle.

The determination of gel fraction is an efficient way to evaluate the crosslinking polymeric matrix. In this test, the hydrogels obtained a high content of gel, indicating a high reticles formation between polymeric molecules. The obtained results of PVP and PVA gel fraction were at about 84% and 74%, respectively.

According to a similar study realized by Zhao and colleagues (2003), the PVA hydrogel associated with carboxymethylated chitosan irradiated at 20kGy dose showed gel fraction at about 88%, and this result is very close to that obtained in this work, which was approximately 84%.

The obtained results in the swelling test showed that PVP and PVA hydrogels have a good capacity of swelling and in the first 6 hours observed a rapid swelling of both hydrogels. It can be observed in Fig. 1 that the PVP hydrogel showed a higher swelling capacity than PVA, getting in 24 hours a difference of 25%.

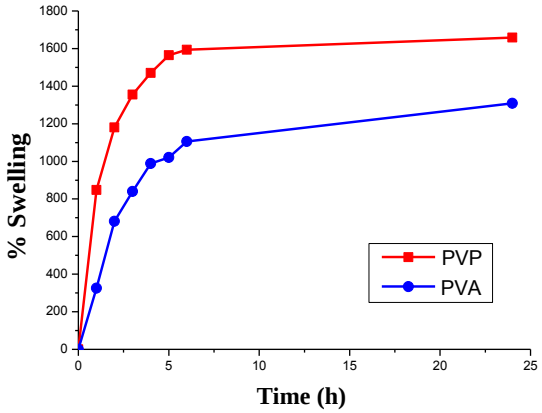


Figure 1. Swelling profile of PVP and PVA hydrogel matrices

Ajji and colleagues (2005) in a study about the production of PVP hydrogels dressings using different concentrations of PVP and PEG, irradiated by gamma radiation at a dose of 25kGy showed similar behavior to that obtained in this work. The swelling capacity could increase about 20 times their initial weight.

The *in vitro* cytotoxicity test allows analyzing the toxicity or biological reactivity induced by tested material in cell cultures. According Eisenbrand and colleagues (2002), *in vitro* cytotoxicity methods detect the ability of a material to cause cell death as a result of damage of cellular basic function and show good correlation with acute toxicity in animals and humans. With the obtained results of DO was calculated the cell viability in relation to the control cells in the test, which was considered 100%. Projecting the values of cell viability in relation to the extract concentrations were obtained cell viability curves as shown in Fig. 2. The cytotoxicity index ($IC_{50\%}$) determines quantitatively the toxic potential of samples, indicating the concentration of extract which causes death in 50% of cell population. If the

cell viability curve cross the 50% viability line is considered cytotoxic and in the intersection is obtained its $IC_{50\%}$.

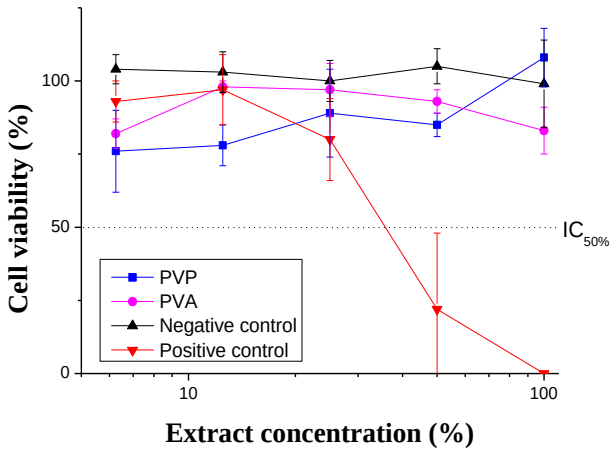
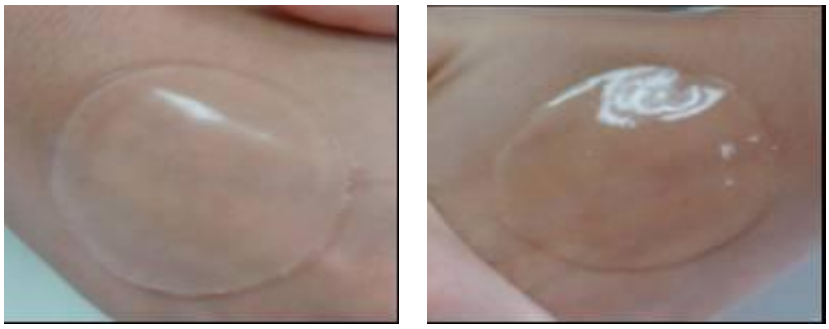


Figure 2. Cell viability curves of PVP and PVA hydrogel matrices in cytotoxicity assay by neutral red uptake method

As previous studies found in literature, the biocompatibility of PVP (Higa, et.al., 1999) and PVA (Burczack et.al., 1996) hydrogels were established. As expected, in this present study, only the positive control showed toxicity with $IC_{50\%}$ equal to 37%, meaning that the positive control extract at a concentration of 37% has killed half the cell population in the assay. Samples of hydrogel matrices showed similar behavior of the negative control, presenting curves above the line of $IC_{50\%}$, therefore both, the PVP and PVA hydrogels showed no cytotoxic effect. This means that these hydrogels were biologically safe, fulfilling one of the safety requirements.

The PVP and PVA hydrogel membrane devices obtained by incorporation of 3% of Arct'Alg® were evaluated visually and by touch as shown in Fig. 3. The criterion for considering the hydrogel membrane devices appropriate or not to be subjected to the release kinetics was the formation of a homogeneous membrane, transparent, flexible and soft, with mechanical properties suitable for handling.



PVA device PVP device

Figure 3. PVA and PVP hydrogel membrane devices

The *in vitro* release kinetic was performed to determine the citrullyl-arginine release profile from the obtained PVP and PVA devices.

The quantification of citrullyl-arginine released was obtained by comparing the chromatogram peaks with the peak area of citrullyl-arginine standard solution (0.2 mg/mL). Arct'Alg® is an extract composed of various compounds and the dipeptide citrullyl-arginine is its main constituent, and was used for determination of Arct'Alg® release.

The peak area of citrullyl-arginine was obtained by injecting 5µL of standard solution and its chromatogram is shown in Fig. 4 with peak in the retention time of about 8 min.

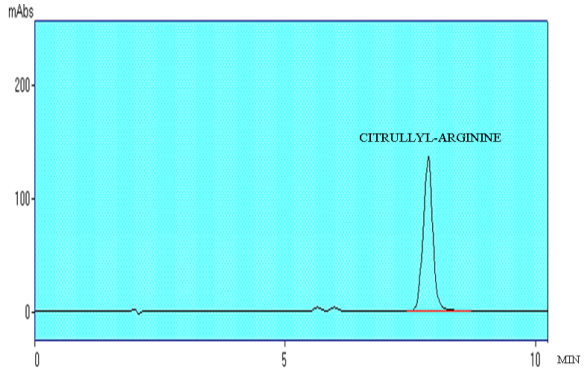


Figure 4. Chromatogram of citrullyl-arginine standard solution

The chromatograms obtained after 1 hour of release from PVP and PVA devices can be seen in Fig. 5 and 6, respectively.

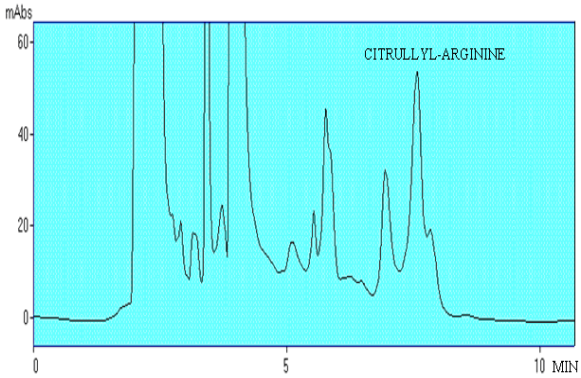


Figure 5. Kinetic release chromatogram of Arct'Alg® from PVP device

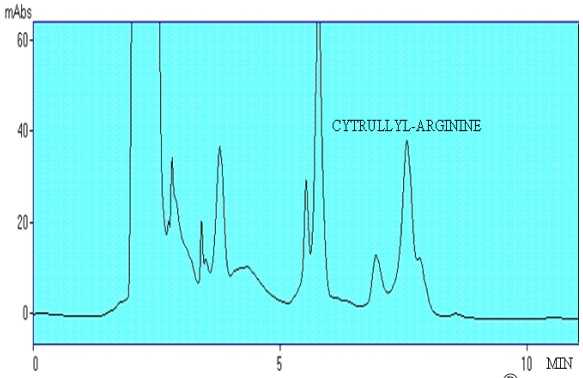


Figure 6. Kinetic release chromatogram of Arct'Alg® from PVA device

In Fig. 5 and 6 is observed citrullyl-arginine peak with retention time at about 8 min, similar to standard.

To prove that the peak obtained in the chromatograms of PVP and PVA devices at around 8 min is really citrullyl-arginine, this dipeptide was incorporated in PVA hydrogel membrane. In the kinetic release chromatogram showed similar profile as that obtained for PVA device with Arct'Alg® (Fig. 7 and 6).

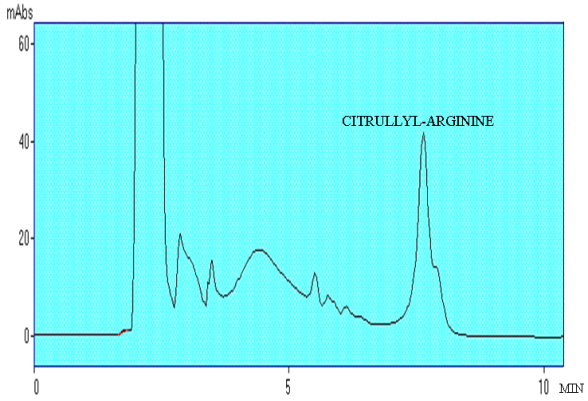


Figure 7. Chromatogram of citrulil-arginine from PVA device

The amount of citrullyl-arginine standard injected corresponds to 1µg which provided a peak area of 1834301. So, it was possible to calculate the concentration of citrullyl-arginine in the aliquots of Arct'Alg® kinetic release of devices and the results are shown in Tables 1 and 2.

Table 1. Results of citrullyl-arginine released from PVP device.

Device	Release time (h)	Peak area	Citrullyl-arginina (µg/mL)
PVP	1	1102231	6.009
	2	938004	5.114
	3	916417	4.996
	4	824082	4.493
	5	839009	4.574
	6	828229	4.515
	7	818832	4.464
	24	842494	4.593

The citrullyl-arginine release profile of PVP and PVA devices presented in Fig. 8 show that the maximum citrullyl-arginine release was in the first hour, with no increase in the release level up to 24 hours.

In a study of Sen and colleagues (2007) they observed that in the hydrogel preparation by gamma radition utilizing soluble PEGs of low molecular weight showed no crosslink and consequently increasing pores size in the formed hydrogel, so facilitating the active agent diffusion. This study may explain the maximum citrullyl-arginine release in the first hour of the test.

Table 2. Results of citrullyl-arginine released from PVA device.

Device	Release time (h)	Peak area	Citrullyl-arginina (µg/mL)
PVA	1	667615	3.639
	2	653028	3.560
	3	626414	3.415
	4	621342	3.387
	5	616679	3.362
	6	503148	2.743
	7	509028	2.775
	24	493516	2.690

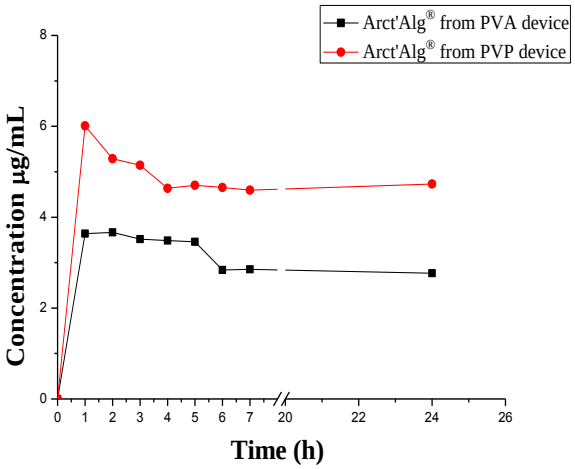


Figure 8. Citrullyl-arginine release profile from PVP and PVA hydrogel devices in cumulative kinetic release

To obtain the released Arct'Alg® percentage was calculated the amount of citrullyl-arginine contained in each membrane. Arct'Alg® has 7.5% of dry residue which 7% of this is citrullyl-arginine. It was incorporated 3% of Arct'Alg®, so, based in this information, the amount of citrullyl-arginine contained in each membrane was 0.79 mg.

Table 3. Kinetic release results of PVP hydrogel device

Hydrogel Device	Release Time (h)	Citrullyl-Arginine (mg)	(%)	Released ARCT'ALG® (%)
PVP	1	0.421	53.3	53.3
	2	0.370	46.8	46.8
	3	0.359	45.6	45.6
	4	0.324	41.1	41.1
	5	0.329	41.6	41.6
	6	0.325	41.1	41.1
	7	0.321	40.7	40.7
	24	0.330	41.6	41.6

In the first hour the citrullyl-arginine amount released from PVP device was about 0.421 mg, i.e, about 53.3% of total active agent immobilized, it means that the same percentage amount of Arct'Alg® was released too. The same calculation was used for the other aliquots and the results are presented in Tables 3 and 4. Probably this released amount was the total active principle in the hydrogel membrane devices. It could be consequence of irradiation process which could therefore cause damage to the Arct'Alg® components.

Table 4. Kinetic release results of PVA hydrogel device

Hydrogel Device	Release Time (h)	Citrullyl-Arginine (mg)	(%)	Released ARCT'ALG® (%)
PVA	1	0.255	32.2	32.2
	2	0.256	32.4	32.4
	3	0.246	31.2	31.2
	4	0.244	30.9	30.9
	5	0.242	30.6	30.6
	6	0.198	25.2	25.2
	7	0.199	25.3	25.3
	24	0.194	24.5	24.5

In the cytostimulation test was verified the cell viability increasing. The fibroblast present in skin tissue is the first cell involved in skin repair process. The processes of tissue regeneration are characterized by a high production of collagen and spontaneously synthesize nitric oxide (NO). NO is produced from arginine by a reaction mediated by NO syntethase enzyme (Witte et al., 2002).

The Arct'Alg® has in its composition the citrullyl-arginine dipeptide which has cytostimulation action as biological property, very important to skin repair process. Therefore the *in vitro* released Arct'Alg® was evaluated for its cytostimulation activity on fibroblastic cells by neutral red incorporation uptake technique.

Table 5. Results of cell viability of Arct'Alg® released from devices

Microplate number	Cell viablility (%)
1	100 ± 12
2	122 ± 8
3	183 ± 14
4	108 ± 11

The released Arct'Alg® from PVP device (microplate 3) showed a significant increase in cell growth at about 80%. The cells in total nutritional condition (microplate 2) showed a growth around 22%. In microplate 4 related to PVA device, the result of viability was very close to the control cell in the test, no significant increase in the number of cells. The results are shown in Table 5 and in the Fig.9.

These results demonstrated that in the *in vitro* test of cytostimulation the PVP device still maintained an appropriate amount of Arct'Alg® to promote the growth of fibroblast cells. Even after irradiation at 25 kGy the active agent showed cytostimulation property.

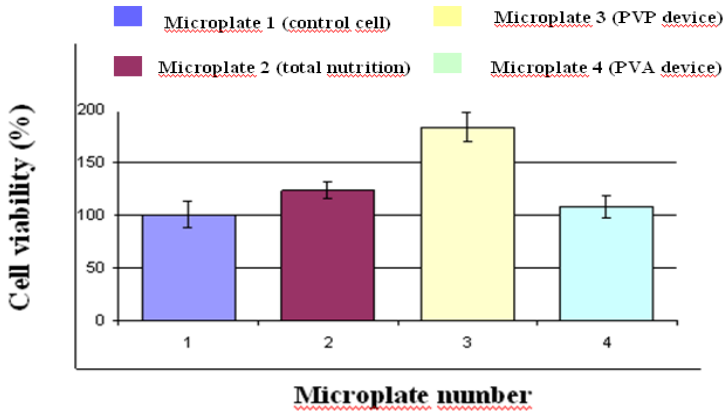


Figure 9. Cell viability of Arct'Alg® released by PVP and PVA devices in the *in vitro* cytostimulation assay

4. CONCLUSION

PVP and PVA hydrogels showed non-toxic effect on the cytotoxicity test and presented a high degree of crosslinking and swelling showing appropriated characteristics to be used as matrices to compose a delivery system.

Even though PVP and PVA hydrogel membrane devices were irradiated at 20 and 25 kGy dose, it was observed Arct'Alg® release at about 50 and 30% respectively, indicating partial radiation damage on citrullyl-arginine.

In the cytostimulation test PVP device released an appropriate concentration of Arct'Alg to promote the cell growth, indicating that PVP device was more efficient than PVA device, so the most appropriate device for use in skin repair processes.

Studies will be continued to incorporate the Arct'Alg® by the swelling capacity of hydrogel matrix, in order to obtain higher incorporation of active agent with cytostimulation action.

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