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Radio-synthesized polyacrylamide hydrogels for proteins release

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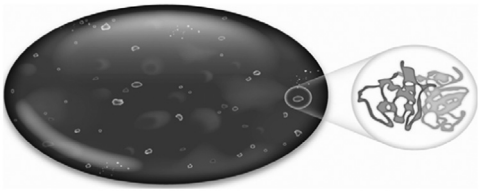
HIGHLIGHTS

- Method for synthesis of polyacrylamide (copolymer) hydrogels using γ-irradiation.
- Polyacrylamide hydrogels suitable for protein loading and release.
- Controlled release of proteins and bioactivity maintenance.
- Noncytotoxic profile observed for these protein containing hydrogels.

GRAPHICAL ABSTRACT



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ABSTRACT

The use of hydrogels for biomedical purposes has been extensively investigated. Pharmaceutical proteins correspond to highly active substances which may be applied for distinct purposes. This work concerns the development of radio-synthesized hydrogel for protein release, using papain and bovine serum albumin as model proteins. The polymer was solubilized (1% w/v) in water and lyophilized. The proteins were incorporated into the lyophilized polymer and the hydrogels were produced by simultaneous crosslinking and sterilization using γ-radiation under frozen conditions. The produced systems were characterized in terms of swelling degree, gel fraction, crosslinking density and evaluated according to protein release, bioactivity and cytotoxicity. The hydrogels developed presented different properties as a function of polymer concentration and the optimized results were found for the samples containing 4–5% (w/v) polyacrylamide. Protein release was controlled by the electrostatic affinity of acrylic moieties and proteins. This selection was based on the release of the proteins during the experiment period (up to 50 h), maintenance of enzyme activity and the nanostructure developed. The system was suitable for protein loading and release and according to the cytotoxic assay it was also adequate for biomedical purposes, however this method was not able to generate a matrix with controlled pore sizes.

1. Introduction

Hydrogel systems may be nanostructured by controlling pore size (Pacios et al., 2006) in order to achieve desirable physico-chemical and mechanical characteristics and also release profiles providing an optimized environment for such substances.

Polymers derivate from acrylates, vinyl alcohol, vinylpyrrolidone and specially acrylamide are currently selected for hydrogel synthesis mainly due their chemical properties, biocompatibility and low toxicicity being suitable to constitute pharmaceutical delivery systems (Rosiak et al., 1983; Rosiak and Ulanski, 1999; Peppas et al., 2000).

The selection of papain and BSA as a model proteins was based on well described structure for BSA and properties of wound debridement for papain (Gurung and Skalko-Basnet, 2009; Naddaf et al., 2010). Polyacrylamide was chosen due to its superabsorbent properties and its recognized applicability in biochemical processes (Bardajee et al., 2008).

Therapeutic proteins and proteolytic enzymes are currently loaded in hydrogels for wound treatment. Although these products have shown efficacy, problems regarding stability of the biomolecule in the pharmaceutical form restricts its use.

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This work aimed the synthesis of a nanostructured PAAM membrane suitable for protein release crosslinked by ionizing radiation using papain and bovine serum albumin (BSA) as protein models in order to evaluate the influence of pore size in release rate and the enzyme stability in this encapsulating system.

2. Experimental

2.1. Materials

Commercially available non-ionic grade polyacrylamide (PAAM) (molecular weight 2.5×10^7 g mol^{-1}) was obtained from Produquímica (Brazil) with 30% of hydrolysis (copolymer of acrylamide and acrylic acid); Papain (30,000 USP ml^{-1}) was purchased from Merck (Brazil); Bovine Serum Albumin (BSA) and *n*-benzoyl-DL-arginine-*p*-nitroanilide was purchased from Sigma Aldrich (Germany). Milli *Q*™ water was used in all experiments. All chemicals were of analytical grade.

2.2. Methods

2.2.1. Hydrogel synthesis

Sample of PAAM was solubilized in water to reach a polymer concentration of 1% (w/v) and then submitted to freezing drying process. Aliquots of water (control membranes) or protein solution were added to lyophilized powder to reach final polymer concentrations of 4, 5, 6 and 10% (w/v). Radiation induced simultaneous crosslinking and sterilization (Rosiak and Olejniczak, 1993) using 25 kGy at dose rate of 1.4 kGy h^{-1} was used for hydrogels synthesis. All samples were frozen at −20 °C and irradiated in the presence of solid carbon dioxide.

2.2.2. Protein loading

Papain and BSA solutions were added before irradiation process under the same conditions adopted for the control membranes to reach final concentration of 0.2% (w/v) to papain and 1% (w/v) to BSA.

2.3. Hydrogel characterization

2.3.1. Swelling degree

Samples of PAAM hydrogels (aprox. 1 g) were oven dried until constant weight. The samples were immersed in physiological solution in excess, to remove the uncrosslinked fractions, and at different time intervals the weight was registered until equilibrium was established (constant weight). The swelling equilibrium was calculated according to the following equation

$$S = (W_f - W_i)/W_i \; 100, \tag{1}$$

where W_i is the initial weight (dried sample) and W_f is the Swollen weight (dried sample).

2.3.2. Crosslinking density determination

Crosslinking density was determined according to Flory (1953), Rosiak et al. (1988), Mahmudi et al. (2007). The following equations

$$M_c = -\ln(1-\chi) + \chi + \mu\chi^2 + p \times v/(\chi^{1/3} - 0.5\chi) \tag{2}$$

$$q = w/M_c \tag{3}$$

were applied.

The parameters selected corresponded to $p = 0.77$ g mol^{-1} and $v = 18$ g mol^{-1}. The polymer/solvent interaction parameter $\mu = 0.495$ was based on Mark (1999), χ value was obtained for each sample based in swollen hydrogel data, $w = 71$ g mol^{-1} and q corresponded to the crosslinking density values (g cm^{-3}).

2.4. Biological approach

2.4.1. Protein release

Protein release was measured by immersion of the matrices in buffer physiological solution pH 6.0 at 37 °C. Aliquots (2 ml) were collected as a function of time (0–50 h) and characterized in terms of protein content for both proteins and bioactivity for papain.

2.4.2. Protein content determination

Drug-loaded hydrogels were immersed in phosphate buffer pH 6.0 and the aliquots were taken and measured by UV–vis spectrophotometry ($\lambda = 280$ nm) on a Hitachi spectrophotometer model Cary 1E.

2.4.3. Enzymatic activity assay

Papain activity was measurement using *n*-benzoyl-DL-arginine-*p*-nitroanilide (Erlanger et al., 1961) as a substrate at 40 °C using phosphate buffer pH 6.0 containing cysteine on a microplate and assayed on a Elisa reader model Multiskan EX Microplate Photometer Thermo Scientific ($\lambda = 405$ nm).

2.4.4. Citotoxicity

Balb 3T3 cells (CCL-163™; ATCC, Manassas, VA, USA) were cultured at 37 °C in 5% (v/v) CO_2 and 97% humidity in complete DMEM tissue culture medium supplemented with 10% (v/v) fetal bovine serum, 100 IU penicillin/100 μg ml^{-1} streptomycin and 4 mM L-glutamine. The assay was conducted according to Esteves-Pedro et al. (2011). Briefly the effects of membrane extract (ISO 10993/EN 30993, 1992) on cell proliferation were added to 96-well plates containing 15,000 cells, followed by addition of eight different concentrations of membrane extract (100 to 0.8% (v/v)). The cytotoxicity evaluation was carried out by using the CellTiter 96R Aqueous Non-radioactive Cell Proliferation Assay and the amount of formazan produced by the cells was determined by measuring sample absorbance at 490 nm with a spectrophotometer (SpectraMaxR 190—molecular devices).

3. Results and discussion

3.1. Hydrogel synthesis

The PAAM hydrogels were prepared by freeze-drying in order to allow a much higher proximity of polymer molecules since this condition promotes polymer crosslinking. Moreover, low temperatures are suitable to encapsulate proteins since under low temperatures protein degradation is minimized, favoring protein stability and integrity.

Radiation induces chain scission and crosslinking depending among other factors on the temperature and chemical structure (Schnabel, 1981). Under low temperatures, as reported by Ozmen et al. (2007) chain scission and radiation indirect effects—water radiolysis were impaired. Regarding the synthesis of PAAM hydrogels, the samples irradiated at room temperature showed no membrane formation; on the other hand it showed an intense decrease in viscosity. This was an evidence of predominance of chain scission over crosslinking. Such fact was not observed for the samples irradiated under low temperatures, where membrane formation occurred. Under freezing conditions the polymer molecules were excluded from ice crystals, therefore they were in close contact each other. Under this special condition, the crosslinking of PAAM solutions were achieved and these membranes were then submitted to characterization.

Table 1
Swelling degree values obtained for the assayed membranes.

PAAM (%)	Maximum swelling degree (%)		
	Control	Papain	BSA
4	2818 ± 176	187 ± 35	287 ± 85
5	2746 ± 190	327 ± 77	320 ± 93
6	1773 ± 145	228 ± 99	327 ± 74
10	2925 ± 182	413 ± 102	396 ± 80

Table 2
Crosslinking density of PAAM hydrogels at different concentrations.

PAAM concentration (%)	Crosslinking density (mol cm^{-3})		
	No protein	Papain	BSA
4	2.05×10^{-2}	8.54×10^{-1}	8.67×10^{-1}
5	1.10×10^{-2}	3.87×10^{-1}	3.92×10^{-1}
6	2.52×10^{-2}	6.45×10^{-1}	7.43×10^{-1}
10	5.0×10^{-2}	2.70×10^{-1}	2.88×10^{-1}

3.2. Hydrogel characterization

According to Table 1, under the concentration range, no direct correlation could be established between polymer concentration and swelling properties as minimum changes were observed among the membranes. One possible explanation is that the condition of proximity of PAAM molecules was maintained in spite of the hydration procedure with physiological solution. Almost the same behavior was observed in presence of protein. A small increase of swelling was observed for papain and negligible increase was observed for BSA. It is relevant to note that the addition of protein led to an intense decrease in maximum swelling degree.

As observed in Table 2, the presence of protein led to an increase in crosslinking density. The profile observed was specific for each polymer concentration, although higher values were observed for membranes containing BSA comparing to membranes containing papain. This increase in crosslinking density could be attributed to attractive electrostatic forces between proteins and acrylic moieties.

The selection of such route for crosslinking determination was based on specific properties of the developed material on a compound based approach. The polymer solution was not a real solution, based on two aspects: the polymer high molecular weight (2.5×10^7 g mol^{-1}) and the fact that the material was irradiated at frozen state.

A possible explanation for the differences observed in crosslinking degree between the protein containing membranes is related to biochemical composition of the assayed proteins. BSA is constituted by a single polypeptide chain of 583 amino acids folded into three homologous domains (Cartel and HO, 1994). Papain, on the other hand, is composed by 112 amino acids, folded into two domains (Kamphuis et al., 1984). Based on this data, there are remarkable differences in terms of charges, such as the presence of cationic and anionic side chains of amino acid of each protein.

Gel fraction experiments revealed that PAAM membranes presented crosslinking values above 70%, specifically 73–91% indicating that irradiation at 25 kGy under freezing conditions allowed higher levels of polymer crosslinking, while at room temperature we observed predominant chain scission.

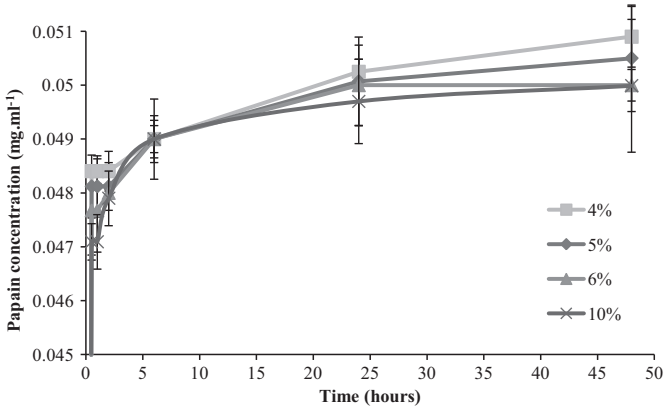

Fig. 1. Papain release in function of concentration (%) and time for different PAAM concentrations (4–10% w/v).

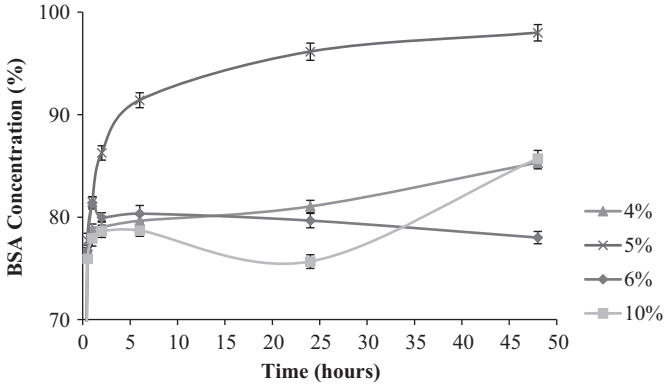

Fig. 2. BSA release in function of concentration (%) and time for different PAAM concentrations (4–10% w/v).

3.3. Protein release

A burst effect (Fig. 1) was observed for the papain samples at the beginning of the exposition (0–2 h). This fact occurred due to the disposition of some papain molecules in the membrane surface, which were quickly released. The remaining particles entrapped in the hydrogel structure allowed constant release that remained up to 50 h. Moreover, the release profile indicated 4% (w/v) as optimum PAAM concentration for papain release since the release profile was the only that increased as function of time (Fig. 1).

Regarding the membranes with BSA (Fig. 2), a less predominant burst effect was observed in the first five hours, instead of two, as occurred for the membranes with papain. In this case, the release profile was gradual and increased as function of time.

The release profile for BSA indicated 5% (w/v) as optimum PAAM concentration considering the amount of protein released. At 4% (w/v) the smallest amount of protein was released and specifically at 10% (w/v) and 6% (w/v) of PAAM an intermediate release was noted. No direct correlation was observed in this case (Fig. 2).

3.4. Papain bioactivity

The bioactivity profile revealed that all membranes presented retained bioactivity which was more pronounced for the membrane containing 4% (w/v) of PAAM. Membranes containing 5% (w/v) and 6% (w/v) PAAM presented similar behavior. Changes in such profile were related to polymer concentrations considering that at minor concentrations (4% w/v) the enzyme released was more active if compared to membranes at higher PAAM concentrations. On the other hand, at higher concentrations – 10% (w/v) – no

relevant changes in bioactivity were observed during the experiment period. At 10% (w/v) PAAM the residual bioactivity maintained lower values; however a continuous profile was observed.

The previous information indicated that the most suitable environment for enzyme loading corresponded to 4% (w/v) PAAM concentration.

3.5. Cytotoxicity evaluation

Under the assayed conditions in accordance with ISO 10993/EN 30993, 1992 the membranes did not present any cytotoxic effects over Balb 3T3 cells, revealing that irradiation at 25 kGy did not generate or led to the formation of toxic residues, as well as the inclusion of proteins in the system, highlighting the membranes potential as biomaterials.

4. Conclusion

The radio-synthesized method to produce PAAM hydrogels was adequate for the membrane preparation; however, this method was not able to generate a matrix with controlled pore sizes. Protein release was controlled by the electrostatic affinity of acrylic moieties and proteins. The system was suitable for protein loading and release and according to the cytotoxic assay it was also adequate for biomedical purposes.

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