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Doxorubicin-Loaded Squid Pen Plaster: A Natural Drug Delivery System for Cancer Cells

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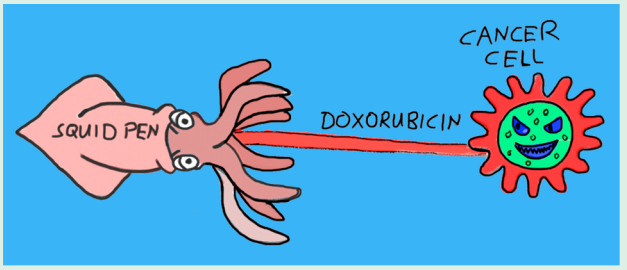
ABSTRACT: The native structure of the β-chitin in the gladius (squid pen) of *Loligo vulgaris* squid can be used as a natural plaster to entrap and release a model drug, doxorubicin, in a targeted and controlled way. Local pH determines the protonation state of the doxorubicin molecules, controlling the two phenomena. Confocal microscopy shows that doxorubicin is uniformly embedded in the β-chitin squid pen and is not simply adsorbed on its surface. Coculture with HeLa cells reveals that the β-chitin squid pen plaster is perfectly biocompatible, while when it is loaded with doxorubicin it shows high cytotoxicity toward the cancer cells. The drug, once released, rapidly accumulates inside the cells. In conclusion, the native structure of a β-chitin squid pen can be potentially applied as a "green" pH-responsive drug vehicle for controlled release.



KEYWORDS: *waste repurposing, chitin, squid pen, doxorubicin, drug delivery system, natural plaster*

INTRODUCTION

Chitin is a widely diffused polymer of β-(1−4)-linked *N*-acetyl-D-glucosamine.[1] It attracts significant industrial interest, thanks to its potential application in a variety of fields, such as cosmetics, agriculture, wastewater treatment, functional food, drug delivery, electronics, regenerative medicine, and catalysis.[1−16] Chitin is found in two main polymorphic forms.[1] The molecular chains constituting α-chitin are arranged in an antiparallel mode and bound by strong intermolecular hydrogen bonding.[1] Oppositely, a parallel chain packing characterizes β-chitin, and intermolecular forces between the chains are weaker than those in α-chitin.[1] This structural organization makes β-chitin more susceptible to swelling, and indeed its natural form is a crystalline hydrate.[1] The most important sources for the extraction of α-chitin, which is the most diffused form commercially available,[17] are represented by the wastes of the seafood industry, constituted mainly by shells of crabs and shrimps. In recent years, the possible technological applications of β-chitin have been attracting growing interest[18−27] and have motivated its extraction from waste products. The richest source of β-chitin is squid pens. From a structural point of view, β-chitin has the unique ability to incorporate in the crystal lattice small molecules different than water, such as alcohols or aliphatic amines.[28,29] An additional advantage in the use of the squid pen chitin is that it requires fewer steps than α-chitin to be processed.[22,23,30,31] In fact, the squid pen (i) does not require a procedure for demineralization because of the trace content of inorganic components in its structure; (ii) does not require a decolorization step to remove leftover pigments and obtain a colorless material; and (iii) the removal of the protein is simpler than for the α-chitin of crustaceans.

The squid pen is a chitin material of dimensions of several square centimeters, and this allows us to cut strips having the size of commercial plasters. Besides this practical aspect, the structure and chemistry of β-chitin from squid pens are in favor of its utilization as plaster for drug delivery. Differently from α-chitin, that contains intermolecular hydrogen bonds among molecular planes, β-chitin swells about 800% when soaked in water solutions.[21] This allows us to easily entrap and release drugs within its tridimensional ultrastructure. Chitin and chitosan are used as a delivery system for doxorubicin (DOX),[32−42] a model drug.[43] The use of chitin/chitosan as drug delivery systems usually requires their transformation in hydrogels, sponges, films, fibers, membranes, powders, beads, or micro-/nanoparticles, thus making their processing expensive, time demanding, and environmentally unfriendly. In this paper, we took advantage of the native structure of the *Loligo vulgaris* squid pen[21,22] to obtain a natural plaster able to entrap and release DOX in a targeted and controlled way.[44]

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The use of renewable and abundant natural resources for the production of technologically innovative materials is one of the most important challenges for materials science.[45−53] The unique 3D architecture of natural scaffolds opens new perspectives for their technological applications. Our approach exploits the intrinsic proprieties of the pristine chitin material, preserving its structure and hierarchical organization at the maximum extent, and gives it an innovative technological application. Free-standing films show a great potential as platforms for delivering bioactive molecules.[54,55] They can be attached on the pathological area of interest for the local controlled release of drugs.[54,55] Several chitin-based dressing materials have already been developed in wound-healing applications. Chitin, combining its beneficial intrinsic properties with the possibility to be used as a drug delivery system, shows a great potential for wound management.[56]

MATERIALS AND METHODS

Materials. Squid (*Loligo vulgaris*) pens were washed several times with deionized water to remove soluble materials. Then, they were air-dried. Clean squid pens were stored in deionized water containing sodium azide.

Squid Pen Treatment. Squid pens were first cut in slices (5 mm apart from the central part of the blade) about 1 cm wide and then were refluxed in 1.0 M NaOH aqueous solution for 2 h to remove proteins. The treatment caused also the hydrolysis of about 5% of chitin's acetyl groups, as we reported in a previous work.[22] After the alkali treatment, the slices were washed thoroughly with distilled water until the pH was close to the neutrality, air-dried, and weighted.

Squid Pen Drug Loading. DOX solutions were obtained by solubilizing DOX (98%, LC laboratories) in Milli-Q water. HCl or NaOH solutions (Sigma-Aldrich) were used to adjust the pH, which was measured with a pH meter (Basic 20, Crison). Pieces of squid pen (1 cm × 1 cm) were soaked in 2 mL of DOX solution and kept at 37 °C for 2 days on a rocking table. Each experiment was replicated 3 times.

Evaluation of DOX Loading. The loading of DOX was evaluated by using UV−vis spectroscopy (Varian Cary 300 Bio UV−vis) to measure the absorbance of the DOX solution (λ = 498 nm) before and after squid pen soaking. The amount of DOX loaded into the squid pen was then calculated as the difference in the absorbance value before and after the squid pen soaking, using as a molar absorption coefficient ε = 11 341 M^{-1} cm^{-1}, which was obtained using a calibration curve.

Evaluation of DOX Release. The kinetics of DOX release was evaluated by UV−vis spectroscopy (Varian Cary 300 Bio UV−vis). The release was studied both in a 0.1 M citrate buffer solution at pH 5.6 and in 0.1 M PBS (Lonza) at pH 7.4, following the absorbance of DOX (λ = 498 nm). The quantity of released DOX was obtained by numerical calculation after obtaining the molar absorption coefficient (ε_{cit} = 10 364 M^{-1} cm^{-1}, ε_{PBS} = 10 401 M^{-1} cm^{-1}) using a calibration curve. The absorbance was normalized on gladius weight and volume of solution used.

***In Vitro* Cell Culture Model.** HeLa cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium; Gibco-Life Technologies Corporation) supplemented with 10% FBS (fetal bovine serum; South-America), 2 mM L-glutamine, and 50 U/mL of penicillin/streptomycin. Cells were kept in an incubator at 37 °C, 5% CO_2, 21% O_2. Cells were seeded in 24-well plates at 40 × 10^3 cells/well and were allowed to grow for 8 h in the complete medium before being exposed to DOX/squid pen and pure squid pen: this point is considered time 0 h. Acquisitions were carried out at 0 h, 24 h, 48 h, 72 h, and 96 h. Cell viability was assessed by using the Trypan Blue exclusion method.

Uptake of DOX by HeLa Cells. The uptake of doxorubicin released from the DOX/squid pen was followed using a Nikon inverted optical microscope TiE equipped with a fluorescence module. The cells were cultured in an incubator at 37 °C, 5% CO_2 for 24 h before the experiment as previously described. The cells were placed under the microscope, and a time-lapse experiment allowed following the uptake of DOX by exploiting the fluorescence of the drug. The emission was detected using a FITC filter from Nikon instruments.

RESULTS AND DISCUSSION

We tested the potential use of β-chitin from the squid pen as plaster for drug release using the anticancer drug DOX as a model. This choice was performed due to the therapeutic relevance of DOX[43] and its ease of detection.

Effect of the pH on Loading. Due to the importance of the net charge of DOX in the entrapment process, we used 1 mM DOX solutions at two pH values, 8.00 and 8.35. The amino group of DOX in physiological environment is protonated with a pK_a value of 8.22 (Figure 1).

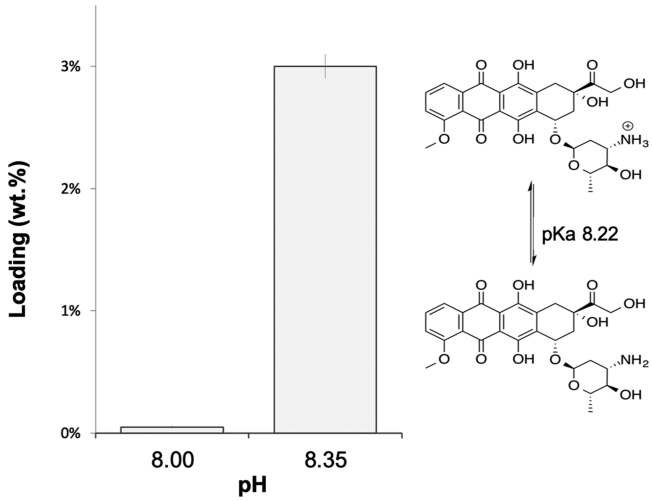

Figure 1. Percentage (wt %) of loading as a function of pH. Inset: DOX protonation equilibrium.

As shown in Figure 1, when the pH of DOX solution is 8, the loading of DOX into squid pens is very low (0.05 ± 0.02 wt %). The same result was obtained at several pH values below 8 (data not shown). When the pH was 8.35, the loading of DOX increased to 3.00 ± 0.10 wt %.

When the pH is below 8.22, the amine group of DOX is protonated, and the molecule has a net positive charge (charge +1, see Figure 1). Electrostatic repulsions among charged DOX molecules prevent DOX loading into the squid pen structure. When pH is above 8.22, DOX molecules are neutral. Thus, there is no electrostatic repulsion among them, and they can accumulate freely in the squid pen. Higher pH could not be investigated because DOX is hydrolyzed at alkaline conditions.

Effect of the Initial Concentration on Loading. The dependence of the amount of DOX entrapped in the β-chitin squid pen by the initial concentration of DOX in solution was evaluated by soaking the β-chitin squid pen in DOX solutions at different concentrations at pH 8.35. As shown in Figure 2, DOX loading has a logarithmic dependence on the initial DOX concentration in solution, allowing us to control the amount of DOX entrapped in the squid pen to reach the optimal loading for the desired use.

Distribution of Doxorubicin within the Squid Pen Plaster. Confocal laser scanning microscopy was employed to

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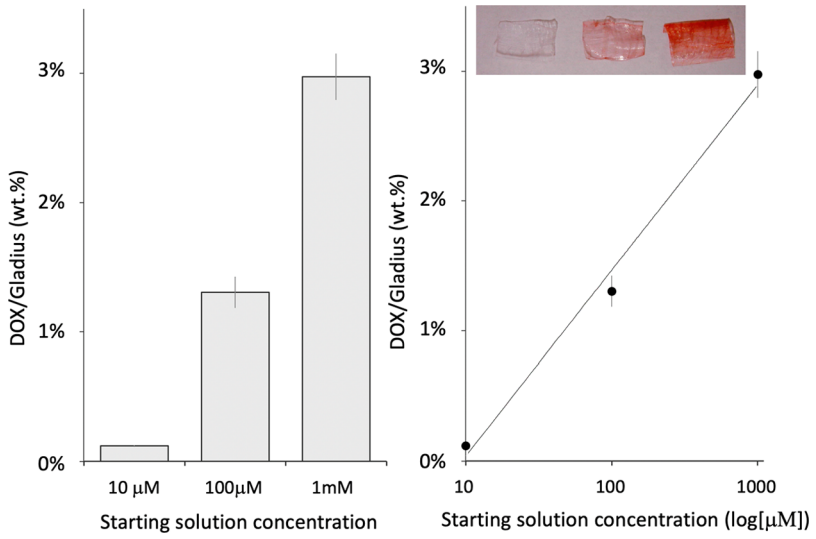

Figure 2. Loading (wt %) of DOX in the squid pen as a function of concentration of starting solution evaluated by the difference of absorbance from the starting and final solution after 2 days. Inset: photo of the squid pen treated with DOX at starting concentration of 10 μM, 100 μM, and 1 mM (from the left), at pH 8.35. DOX/Gladius (wt %) = 0.0062 log[DOX(μM)] − 0.0139.

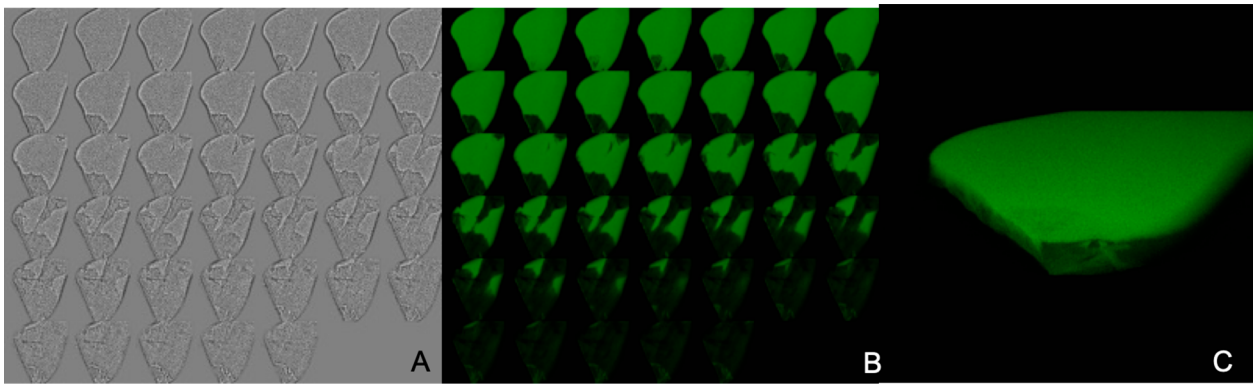

Figure 3. Single *xy* planes obtained (A) in reflection mode and (B) in fluorescence. (C) 3D visualization of a *z*-stacking of the DOX/squid pen treated with 1 mM DOX at pH 8.35. The fluorescence was excited using a 488 nm laser line, and the detection was set up in the 509−650 nm wavelength range in order to capture the DOX emission. For the reflection mode, a detection window of 482−504 nm was used. The size of each image is 375 μm × 375 μm.

investigate the distribution of DOX within the β-chitin squid pen treated with 1 mM DOX at pH 8.35. Figure 3 shows the confocal optical sectioning of the squid pen sample containing DOX. The fluorescence signal is homogeneous in all the captured planes of the squid pen plaster suggesting that the embedding of DOX in the β-chitin squid pen sample is uniform and is not limited to the external surface. The reference squid pen, which did not contain DOX, did not show fluorescence.

Targeted Release of the Doxorubicin by the Squid Pen Plaster. The DOX release kinetics from a DOX/β-chitin squid pen treated with 1 mM DOX at pH 8.35 was measured by UV−Vis spectroscopy at pH 5.6 (0.1 M citrate buffer) and at pH 7.4 (0.1 M PBS buffer). DOX release has a burst within the first 2 h, followed by a slower release. The DOX release from the DOX/β-chitin squid pen (Figure 4) is pH-sensitive and is controlled by the protonation state of DOX molecules, similarly to what was previously observed for the loading.

Because of the acidic extracellular environment of solid tumor tissues, the β-chitin squid pen may represent an innovative plaster for controlled drug delivery. In fact, the

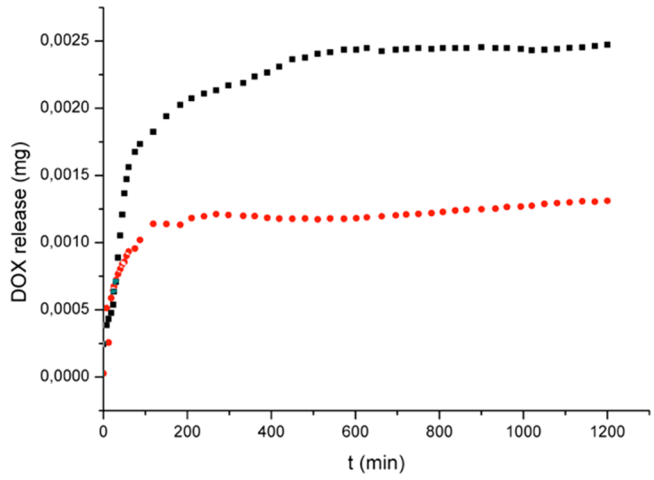

Figure 4. Kinetics of DOX release from the DOX/β-chitin squid pen treated with 1 mM DOX at pH 8.35 in citrate buffer at pH 5.6 (black) and in PBS buffer at pH 7.4 (red)

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DOX release from the DOX/β-chitin squid pen is pH-sensitive and can be triggered by the acidic tumor microenvironment.

***In Vitro* Test of the Doxorubicin-Charged Squid Pen Plaster.** The pharmacological activity of the DOX/β-chitin squid pen treated with 1 mM DOX at pH 8.35 was tested *in vitro* on cell cultures. We cultured HeLa cells in culture medium, in the presence of a squid pen and in the presence of a squid pen loaded with DOX (Figure 5). Cells were counted at different time points, and cell growth was investigated.

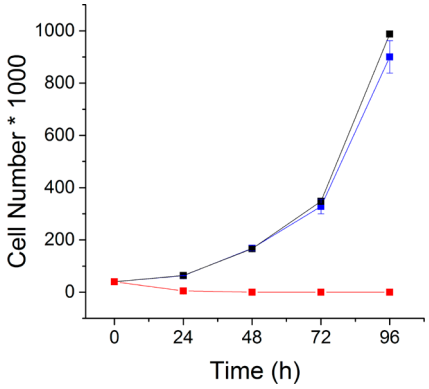

Figure 5. Cellular growth curves of HeLa cells in culture medium (black squares), in the presence of a squid pen (blue squares), and in the presence of a squid pen loaded with DOX (red squares). Time 0 h is the point at which the gladii were inserted in the wells. The values reported were obtained by averaging the cell numbers of three experiments/time points.

The HeLa cell growth curve obtained in the presence of a pristine squid pen is similar to the one obtained in standard control conditions, revealing the biocompatibility of the squid pen. In contrast, all the cells died after 24 h of culturing in the presence of DOX/gladius in the medium. This result shows that the toxicity of DOX is retained once entrapped in the squid pen and that the squid pen is able to release DOX in effective dose concentration. Very importantly, cancer cells may specifically induce the release of DOX from the gladius as a consequence of the decrease of the pH caused by cell metabolism.

Cellular Uptake of the Doxorubicin by HeLa Cells. In order to assess the DOX cellular uptake, the DOX fluorescence signal was measured in HeLa cells after their exposition to the DOX/β-chitin squid pen (Figure 6). The cells were cultured for 6 h in the presence of the DOX-loaded squid pen and were imaged using a fluorescence microscope. As shown in Figure 6, the DOX fluorescence signal was detected in the treated HeLa cells, demonstrating the DOX cellular uptake. The cells cultured in the presence of the control gladius do not show any fluorescence signal.

The molecular distribution of the DOX clearly reveals a rapid nuclear uptake and stronger fluorescence intensity in the nuclei, in accordance with the well-known role of doxorubicin as a DNA intercalator.

CONCLUSIONS

In this paper, we demonstrated that the squid pen can efficiently act as a biocompatible drug delivery system. β-Chitin has the unique ability to incorporate small molecules in the ultrastructural network. The loading and the release of the model drug DOX in the β-chitin squid pen are pH-sensitive and are governed by the DOX protonation state. It is possible to entrap a considerable amount of drug (>3 wt %) inside the β-chitin squid pen and tune the amount of DOX entrapped by selecting the initial concentration of the DOX solution. Confocal microscopy showed that DOX is uniformly embedded into the β-chitin squid pen and that the drug is not simply adsorbed on the external surface. The drug release from the DOX/β-chitin squid pen is pH-sensitive and can be triggered by the acidic environment typical of solid tumor tissues. Coculture with HeLa cells revealed that the pristine β-chitin squid pen plaster is biocompatible, while when loaded with DOX, it shows a high cytotoxicity toward the cancer cells, proving that the activity of DOX is retained once entrapped in the β-chitin squid pen and that the released DOX concentration is higher than the effective dose. The drug, once released, rapidly accumulated in the cell nuclei. Even if the current set of experiments is not enough to support squid pen plasters as a novel drug delivery system with clinical significance, because *in vivo* PK/PD, therapeutic, and toxicity studies are needed, they pave the way to translational applications.

In addition, given that β-chitin squid pens are natural waste products of the fishing industries, their repurposing is a significant advantage for the environment and industry, especially since, if not reutilized, they are a source of environmental contamination and an economic burden for processing factories.[57] Squid pens are available in considerable amounts, and this paper demonstrated that their reuse opens up a plethora of applications ranging from medicine and

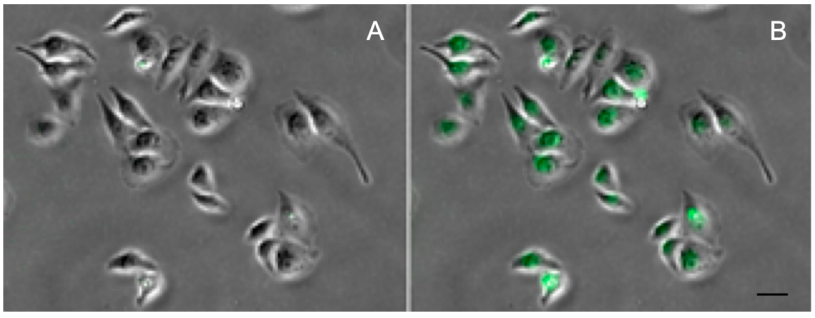

Figure 6. Fluorescence microscopy image merged with optical image of HeLa cells cultured in the presence of the DOX/β-chitin squid pen treated with 1 mM DOX at pH 8.35 (A) at time 0 from the addition of the DOX/β-chitin squid pen in the culturing environment. (B) Time 6 h after the addition of the DOX/gladius. The fluorescence was excited using a mercury lamp, and the excitation/emission was filtered using a FITC cubic filter. Objective used 10×. Scale bar 20 μm.

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pharmacy to food industry and agriculture. In fact, the same approach is exploitable to load the β-chitin squid pen with different drugs, such as anti-inflammatory/antibacterial drugs, or with molecules that need to be released in a controlled manner as pesticides or fertilizers.[58]

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Notes

The authors declare no competing financial interest.

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