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RESEARCH ARTICLE

ANNEXATION OF BIOLOGICALLY ACTIVE COMPOUNDS EXTRACTED FROM PLANTS IN THE LIPID LAYERS OF MICRO BUBBLES FOR THE LOCALIZED TREATMENT OF DISEASES

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ABSTRACT

The article deals with the generation of monodisperse micro bubbles using a microfluidic device based on 3D printing. The micro bubbles play a role of carriers of biologically active compounds to act locally in the chosen region having the ultrasound as the drug-releasing agent from a known frequency. The micro bubbles are generated by the passage of gas (nitrogen) through an emulsion consisting of coconut oil, a surfactant, and water, forming individual outer shell layers consisting of sunflower oil. In the development of the work, micro bubbles with an average diameter of 23.50□m with a dispersion of 1.1% were produced, which characterizes a population with a high degree of homogeneity. The Lupeol used was isolated from *Maytenus acanthophylla (Celastraceae)* plant leaves by phytochemical and spectrometric techniques, including methods in liquid chromatography and 1H and 13C magnetic resonance. The natural product Lupeol is recognized for presenting actions against inflammation, antitumor (prostate cancer), arthritis, diabetes, heart disease, kidney, and liver toxicity. The micro bubbles generated by the technique described above will be applied in in vitro assays to evaluate the behavior of tumor cells in the presence of a population of micro bubbles after collapse caused by the presence of a known ultrasonic frequency and intensity, allowing the interaction of diseased cells with the biologically active component.

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INTRODUCTION

The use of micro bubbles as auxiliary units in the diagnosis of diseases began in the 1980s when they were used to increase the contrast of ultrasound images in echocardiographic examinations. Micro bubbles are small microspheres loaded with a specific gas that has specific acoustic properties which make them very useful as ultra-sonographic contrast agents for diagnostic imaging (Figure 1). The contrast echocardiography, based on the use of ultrasound and micro bubbles, has been used to improve the visibility of endocardial borders and myocardial perfusion analysis. The application of pulses with high mechanical index (IM) using diagnostic transducers results in the cavitation of the micro bubbles and the consequent destruction of the same, releasing the gas thus allowing the analysis of the filing of the contrast of the myocardium. Additionally, ultrasound-mediated destruction of micro bubbles may have therapeutic applications, such as the release of biologically active components (drugs) at specific sites or to accelerate the dissolution of thrombi, also called sleep thrombolysis. Recent developments have demonstrated the feasibility of using micro bubbles as carrier agents for localized delivery of appropriate drugs for the treatment of tumors (Stride, 2009; Stride *et al*, 2009; Pancholi *et al.*, 2008; Unger and Matsunaga, 2002; Lajoline *et al.*, 2016; Guvener. *et al*. 2017). Well known for their use in real-time imaging without dangerous tissue irradiation, ultrasound can also be used to control the timing of drug release when transported by a micro bubble. This type of therapeutic application is a promising treatment modality, particularly in cases where high concentrations of drugs are administered systemically, causing undesirable side effects (Lidner, 2001; Borden, 2002). Overcoming these effects leads to a better quality of life for patients, reducing the possibility of subsequent hospitalization

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during treatment. This work proposes the generation of monodisperse micro bubbles of lipid coating layer having the sunflower oil as a matrix. The micro bubbles are generated through a microfluidic device manufactured through 3D printing. Also, a biologically active compound Lupeol, extracted from the plant *Maytenus acanthophylla*, has been isolated to be attached to the lipid layer of the micro bubble. This compound has proven anti-inflammatory and anticancer properties and widely used in the treatment of uterine tumours in the southwest region of Bahia. The annexation of the component will occur in the generation process. Subsequently, the behaviour of tumor cells in vitro in the presence of Lupeol released after the incidence of irradiation with an ultrasonic field will be studied.

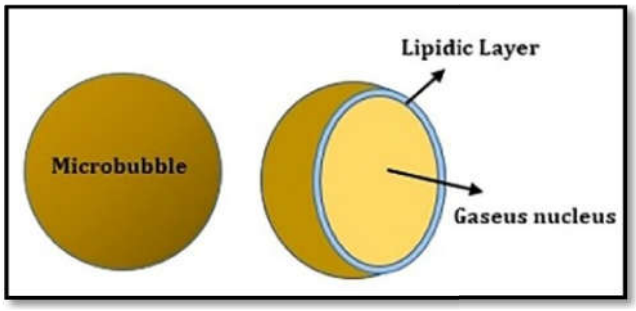

Figure 1. Microbubble structure

PROCEDURES AND METHODS

Generation of micro bubbles

The microfluidic device design developed in the Solid Works environment with the SLDPRT extension and manufactured from a 3D OBJET EDEN 250 printer using the Vero Clear RGD810 transparent resin. Figure 2 shows the fabricated device used to generate micro bubbles.

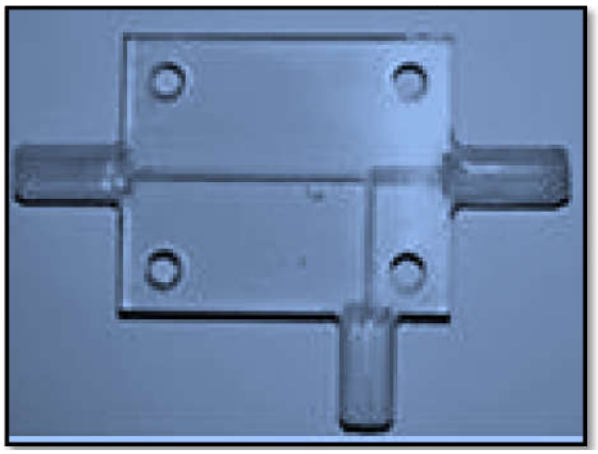

Figure 1. Microfluidics device used to the generation of micro bubbles fabricated using a 3D printer

Figure 3 shows in detail the geometry of the according to internal dimensions. The minimumdiameter of the device channels is 400 μm. This value represents the maximum resolution of the equipment in the manufacture of closed channels, which is a limitation when it is necessary to work with channels of smaller dimensions. This restriction fixed with a micro capillary of maximum outer diameter (body) of 1.0 mm coupled to the device.

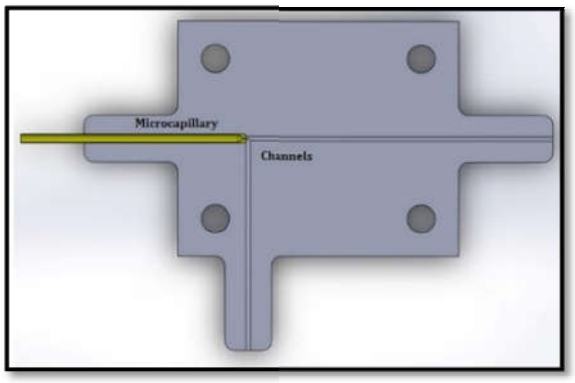

Figure 3. Detail the geometry of the according to internal dimensions

The micro capillary coupled to the device has the function of inflating the microbubble. The opening of the gas phase in the device is connecteddirectly to the size of themicro bubble generated as shown by the empirical equation below (Garstecki, 2010):

$$\frac{D}{\lambda} = 1 + \alpha \frac{Q_G}{Q_L}$$

Where D is the diameter of the micro bubble, Q_G represents the gas phase flow rate, Q_L the liquid phase flow rate, α a proportionality constant and λ represents the opening of the gas phase feed channel. The proportionality constant for this equation depends on the geometric characteristics of the device such as the profile of the channels (circular profile) but is almost independent of the properties of the fluid. In the specific case, α assumes the first order value (where α = 1) Garstecki, 2010). Thus, a micro capillary of 20 micrometers μ of aperture was used to compose the micro bubble generating de-vice. The microfluidic device operates in a two-phase mode as shown in Figure 4. The gas phase feed is performed through a gas cylinder containing Nitrogen (N2), a colorless, odorless and tasteless gas that makes up 78% the atmosphere, with pressure and flow controlled by the devices (SMC AS2000) and (SMC AS2001). Nitrogen has a molar mass of 14.01 g / mol and viscosity of 0.017562551 centipoises (cP). The liquid phase was injected into the capillary system through a hospital syringe pump (ST670-SAMTRONIC INFUSION SYSTEMS). The generation of the micro bubbles were monitored with a high-speed camera (VIS SDK 7.2.1) coupled to a stereo microscope (XTL series - STEREO ZOOM MICROSCOPE). The liquid phase consisted of an emulsion having water, coconut oil and the TWEEN 80 active surfactant in its composition in a mass ratio of 98:1.5:0.5. Figure 4 show too the experimental apparatus used to generate the micro bubbles. Thirty micro bubble images were obtained in one second and processed automatically through a computational tool in a MATLAB® environment, as well as a statistical survey of the entire micro bubbles population generation process.

Obtaining the biologically active compound (Lupeol)

The natural compound Lupeol is a pent acyclic triterpene (TTPC) of the class of lupanes known for its widespread occurrence in several plant families (Connolly and Hill, 2008). Lupeol was isolated from the nonpolar extract of the leaves of *Maytenusacanthophylla* (*Celastraceae)* from phytochemical techniques, including liquid chromatography on silica gel and

spectrometric techniques. *M. acanthophylla* is a medicinal plant, used in the fight against inflammation and uterine cancer in the Southwest of Bahia. The hexane extract prepared with plant leaf and partitioned by liquid chromatography methods provided the compound lupeol as a solid white,with melting at 216.5 °C (DSC). The absorption spectrum in the infrared region showed characteristic bands at 3550 and 1040 cm^{-1} (R-OH) , 3400 cm^{-1} (-CH_2-COH-CH_2-); 2920, 2850, 1470 and 1455 cm^{-1} (-CH_2 and -CH_3); 1640 and 1620 cm^{-1} (RC=CH_2), 1380 and 1360 cm^{-1} (gem-dimethyl group), and 880 cm^{-1}(RC=CH_2).The chemical structure of Lupeol was readily elucidated by ^{13}C and ^{1}H NMR spectroscopy by the hydrogen and olefinic carbons signals ofH_2C-29 and C-20 that resonated at δH 4.69 and 4.57 (br. s, H-29a and b); δ_C109.32 and 150.96, assigned to C-29 and C-20, respectively. NMR spectra also showed the signal of a linked carbinolic (-CH-OH) at C-3 methine group with resonances at δH 3.19 (dd, 11.0 and 5.0 Hz, H-3) and δ_C 79.01 (C-3). Additionally, the signals of five other methane groups, seven methyl groups, ten methylene and six non-hydrogenated carbons, all assigned to the Lupeol structure according to Figure 5 (de Oliveira, 2012), were observed.

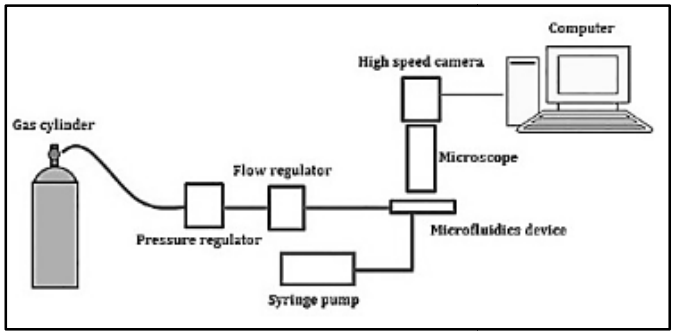

Figure 4. Experimental apparatus used to the generation of micro bubbles

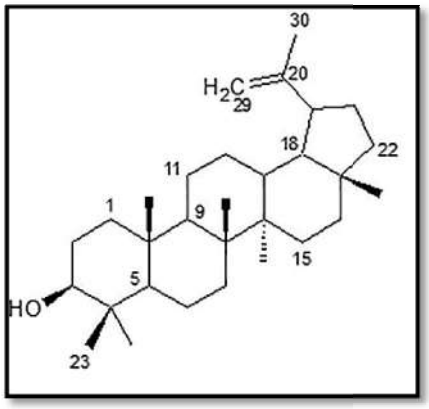

Figure 5. The molecular structure of the TTPC Lupeol isolated from Maytenusacanthophylla

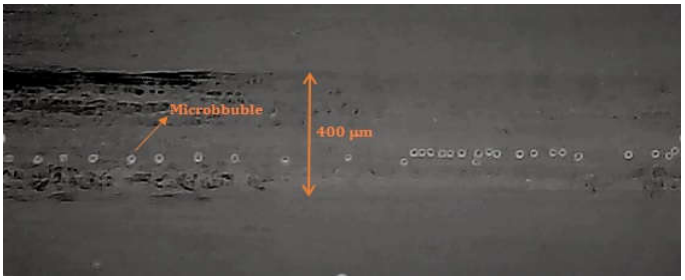

Figure 6. Passage of the micro bubbles downstream of the device channel Micro fluidic T-junction manufactured according to the 3D printing technique (Filho, W.A *et al,* 2012).

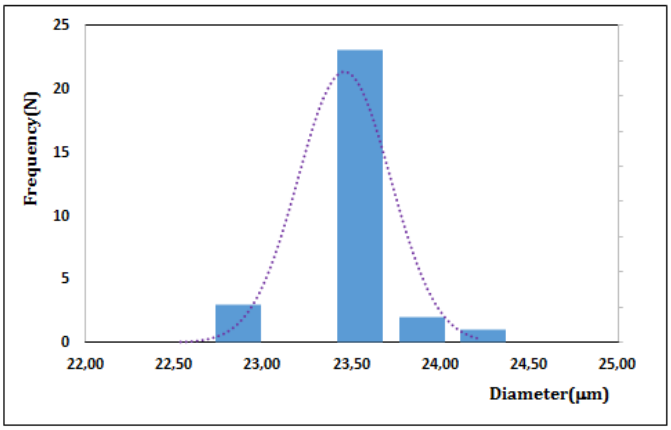

Figure 7. The micro bubbles diameter histogram presented a normal distribution profile. Where, N represents the frequency of the number of events for a gas phase flow (N_2) $Q_{N2} = 7.86\times10^{-4}$µl/s, liquid phase flow and Q_E= 1.38 µl/s and viscosity relative of the liquid phase of ηr = 1.58 mPa.s

Table 1. Diameters and ray's population of thirtymicro bubbles captured in time of 1.0.s

Diameter (µm)	Ray (µm)
23.49	11.75
23.49	11.75
23.51	11.76
22.52	11.26
23.51	11.76
23.51	11.76
23.52	11.76
23.51	11.76
23.51	11.76
23.49	11.75
23.51	11.76
23.51	11.76
23.49	11.75
23.51	11.76
23.52	11.76
23.52	11.76
23.52	11.76
23.52	11.76
23.52	11.76
24.24	12.12
23.63	11.82
23.55	11.78
23.54	11.77
23.64	11.82
23.54	11.77
22.53	11.27
22.52	11.26
23.53	11.77
24.51	12.26
23.51	11.76

Table 2. Quantitative data of micro bubbles generated

Mean Diameter of microbubbles [µm]	23.50
Standard deviation [µ]	0.26
Coefficient of variation [%]	1.1

Obtaining the chemical compound Lupeol by synthetic methods is not an easy task due to its stereochemistry with ten asymmetric centers in the structure, therefore obtaining Lupeol is directed to the natural sources as plants rich in this chemical constituent such as *Crataevanurvala* (Gallo and Sarachine, 2009). Lupeol and derivatives have been reported as active compounds against several tumor cell lines. Published studies show that Lupeol exhibits various pharmacological activities in both vitro as well as in vivo assays. The antitumor effect of

Lupeol may be associated with its potential to modulate metabolic pathways, such as nuclear factor kappa B (NF-κB) and phosphatidylinositol 3-kinase (PI3-K)/Akt (protein kinase B).These activities play a major role in cell signaling during tumor development that may induce apoptosis or regression of tumor growth (Saleem, 2009).

RESULTS

Figure 6 shows the passage of the micro bubbles downstream of the device channel micro fluidic T-junction manufactured according to the 3D printing technique (Filho, W.A *et al*, 2012). The experimental results have demonstrated the effectiveness of the equipment utilized, where four monodis persed microbubbles with average diameter value of 23.50μm, Standard Deviation(SD) of 0.26 μ, and Coefficient of Variation (VC) of 1.1% (see Table 1 and Table 2). Figure 7 shows the micro bubbles diameter histogram presented a normal distribution profile. Where, N represents the frequency of the number of events for a gas phase flow (N_2) $Q_{N2} = 7.86\times10^{-4}$ μl/s, liquid phase flow and Q_E= 1.38μl/s and viscosity relative of the liquid phase of ηr = 1.58 mPa.s.

Conclusion

The results presented show the capacity of the proposed system to generate micro bubbles with average diameter value (Dm) of 23.50μm and Coefficient of Variation (Vc) of 1.1% (see Table 2), which characterizes the monodisperse character of the produced micro bubble population. The micro bubbles with low dispersion are significant and desired characteristic because it increases the effective drug release action,because the frequency of the ultrasonic field has a direct relation to the diameter of the micro bubbles and a narrow range of frequency that potentiates the cavitation and the destruction of the irradiated micro bubbles population. Thus, allowing a significant release of the drug into the desired target. Lupeol for lipophilicity as well as most of the pent acyclic triterpenes and steroid alcohols is considered very high and belongs to the class of non-saponifiable lipids. The log [octanol/water] partition coefficient (Log[P]) is between 8.02 and 9.23, indicating that it is a compound of about one billion times more soluble in n-octanol than in water, which gives Lupeol a characteristic highly lipophilic and permeable to lipid membranes. This physical-chemical characteristic of Lupeol points as a potential candidate to be added to the lipid layer of the micro bubble. Future works will deal with this unfolding, also to study the dynamic behavior of the process of micro bubbles generation in the presence of Lupeol. The behavior of tumors cells in the presence of a population of micro bubbles after collapse caused by the action of a known intensity and frequency ultrasonic field, allowing the interaction of the diseased cells with the biologically active component, will be evaluated.

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