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Preliminary evaluation of the encapsulation of new anti-diabetic sulphonylhydrazone and antitumor \(N\)-acylhydrazone derivatives using PLGA nanoparticles


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Abstract. It has been demonstrated the feasibility of using PLGA nanoparticles to promote the encapsulation of novel anti-diabetic sulphonylhydrazone and antitumor \(N\)-acylhydrazone derivatives. The motivation is to further demonstrate the possibility of long-term release of anti-diabetic as well as higher accumulation of the antitumor derivative by using the nanotechnology-based production. The produced nanoparticles were obtained by the nanoprecipitation method, which revealed to be effective in the encapsulation of the bioactive compounds. The determined sizes were in the range of \(~100\) nm, which are supposed to be suitable for both potential applications. The preliminary experimental data demonstrated the formation of stable nanosystems and further experiments are underway in order to determine the loading content, encapsulation efficiency and release profile of the hydrophobic bioactive compounds.

1. Introduction

Diabetes mellitus and cancer are prevalent global diseases that demand high economic and social costs [1, 2]. The development of diabetes mellitus (DM) has increased enormously during the last years and estimations report that the number of cases may reach 300 million by the year 2025 [3]. In turn, according to the World Health Organization (WHO), the different types of cancer figure among the leading causes of morbidity and mortality worldwide, with approximately 14 million new cases and 8.2 million cancer-related deaths in 2012. The number of new cases is expected to rise by about 70\% over the next two decades [4]. Moreover, type 2 diabetes and cancer share many risk factors including aging, familiar history, obesity, physical inactivity, poor diet, alcohol consumption and smoking [5].

DM is characterized by hyperglycemia as a result of defects in insulin secretion, insulin action or both [6]. Consistently, high blood glucose levels can lead to serious diseases affecting the heart and blood vessels, eyes, kidneys, and nerves. These complications are the main causes of morbidity and mortality in diabetic patients.
The three main types of diabetes are type 1 diabetes, type 2 diabetes and gestational diabetes, being type 2 diabetes the most common one. It usually occurs in adults, however, it has been increasingly seen in children and adolescents [7]. Although the body is able to produce insulin, in type 2 diabetes the amount of insulin may not be sufficient or the body cannot respond to its effects, leading to an accumulation of glucose in the blood. Currently available hypoglycemic agents include sulfonylureas, biguanides, thiazolidinediones, α-glycosidase inhibitors and dipeptidyl peptidase-4 inhibitors. These drugs are widely used to control hyperglycemia, but fail to alter the course of diabetic complications and have limited use due to undesirable side effects and high rates of secondary failure [8].

Cancer is a generic term for a large group of diseases that can affect any part of the body and can be defined as the rapid and unplanned growth of abnormal cells that can invade other parts (healthy) of the body, thus spreading to other organs. The latter process is referred to as metastasis - the major cause of cancer death. For males and females combined, the most common types of cancer that lead to death are: lung, liver, stomach, colorectal, breast, oesophageal, among others [4, 9].

Many types of cancer can be prevented, reduced and/or controlled mainly if early detected but the treatment is still a major challenge. Among the drugs that are in clinical trials and have been approved in the United States we can quote: imatinib mesylate (Gleevec®, Novartis) - that is used for gastrointestinal stromal tumor and chronic myeloid leukemia, gefitinib (Iressa®, AstraZeneca & Teva) used for non-small-cell lung cancer, rituximab (Rituxan®®, Biogen Idec & Genentech) used in the treatment of B-cell non-Hodgkin’s lymphoma and B-cell leukemia and trastuzumab (Herceptin®, Genentech) used in the therapy of breast cancer [10]. It is therefore crucial and strategic the effort to identify new drug candidates that are more potent, selective and less toxic in the therapeutic arsenal against cancer.

The significance of these diseases and the great challenge to discover new therapeutic agents for the treatment of DM, their complications and for treatment of cancer were the major motivation of this work, which aims the development of a nanosystem of two new bioactive compounds - LASSBio-1773 and LASSBio-1735 - which have been found to promote hypoglycemic and antiproliferative activities, respectively. The polymeric nanoparticles (NPs) used in drug-delivery systems have been the subject of extensive research to promote benefits such as protection of encapsulated substances against degradation, reduced side-effects, higher therapeutic efficacy, sustained drug release, specific accumulation, high cellular internalization and long blood circulation half-life [11-14]. In this work we describe preliminary results on the preparation and characterization of LASSBio-1773- and LASSBio-1735- encapsulated into polymeric nanoparticles. These compounds were planned and synthesized in the Laboratory for the Evaluation and Synthesis of Bioactive Substances (LASSBio®) of the Federal University of Rio de Janeiro (UFRJ) and, subsequently, the nanoparticles were prepared at the Laboratory of Crystallography and Structural Characterization of Materials (LCCEM) of the Federal University of ABC (UFABC).

2. Experimental

2.1 Materials and reagents

LASSBio-1471, a sulphonylhydrazone derivative, which was reported as a PPARγ ligand, was shown to be effective in the treatment of diabetic neuropathy in streptozotocin-injected rats [8]. Aiming to optimize the hypoglycemic properties of this prototype, the medicinal chemistry approach of structural modifications using molecular homologation, ring opening and molecular simplification strategies, as displayed in Figure 1, was employed. Then, a new sulphonylhydrazonic compound, LASSBio-1773, was designed. Its activity was evaluated using the murine model of streptozotocin-induced hyperglycemia. One dose of 55 µmol kg⁻¹ of LASSBio-1773 compound was capable to reduce the blood glucose levels (reduction of 53% in 120 minutes).
LASSBio-1773 is also a therapeutic candidate for the treatment of diabetic neuropathy, which is one of the complications resulting from type 2 diabetes. Also, it was able to reduce the thermal hyperalgesia and mechanical allodynia in a diabetic neuropathy model. These results suggest that the presence of the dimetoxyl group, the ester group and the aromatic ring attached to the imine are required for hypoglycemic effect observed in this model [8, 15].

Figure 1. Medicinal chemistry strategies used in LASSBio-1773 synthesis.

The N-acylhydrazone derivative - LASSBio-1735 - was designed from LASSBio-1586, a simple antitumor drug candidate, a Combretastatin A4 (CA-4) analogue, using the N-methylation strategy (see Figure 2). Among the known classes of anticancer agents, the microtubule-targeted antimitotic drugs are considered to be the most important ones. CA-4, which is a natural stilbene isolated from *Combretum caffrum*, is a microtubule-destabilizing agent that binds to the colchicine domain on \( \beta \)-tubulin and exhibits low toxicity. LASSBio-1586 was capable of inhibiting microtubule polymerization and displayed a broad *in vitro* and *in vivo* antiproliferative profile, as well as a better selectivity index than the prototype CA-4, indicating improved selective cytotoxicity toward cancer cells. LASSBio-1586 compound, in turn, presented *in vitro* antiproliferative activity against HL-60 (human leukemia), SF-295 (human glioblastoma), MDA-MB435 (melanoma), and HCT-8 (ileocecual adenocarcinoma) tumor cells and was compared with the data from LASSBio-1586 and CA-4. The homologous compound, LASSBio-1735, was well tolerated, exhibiting a slight increase in the cytotoxic potency against HL-60 (IC\(_{50} = 0.03\) \( \mu \)M) and HCT-8 (IC\(_{50} = 0.54\) \( \mu \)M) tumor cell lines relative to LASSBio-1586 compound [16].
In the synthetic procedures of these compounds, it has been produced solid-phase samples with particles of micrometric sizes. Both LASSBio-1735 and LASSBio-1773 were obtained as white crystalline solids [15, 16]. The FDA approved poly(lactic-co-glycolic acid), PLGA ($M_w = 50,000-75,000$ g mol$^{-1}$; L/G 85:15), was purchased from Sigma Aldrich and used as received. The solvent employed in the preparation of the NPs (acetone) was of analytical grade and was also used as received. The water was pretreated with the Milli-Q® Plus System.

### 2.2 Preparation of the nanoparticles

The PLGA NPs were prepared by the nanoprecipitation approach, as displayed in Figure 3 [17], by using a syringe at a flow rate of $2 \text{ mL min}^{-1}$. The PLGA copolymer (5.0 mg) was firstly completely dissolved in acetone (1.0 mL) at room temperature and subsequently the organic solution was injected into the aqueous phase under stirring. The organic solvent was further removed by dialysis. The final aqueous volume after the nanoprecipitation procedure and solvent elimination was set to 5.0 mL. The LASSBio-1773 and LASSBio-1735 PLGA-loaded NPs were similarly prepared. In such case, 0.5 mg of each bioactive compound was also dissolved in acetone along to the copolymer. The prepared NPs were used immediately or stored at 4°C.
Figure 3. Schematic representation of the preparation of PLGA NPs: (A) organic (polymer or polymer/drug) solution, (B) nanoprecipitation followed by (C) solvent diffusion and (D) solvent elimination via membrane dialysis.

2.3 Characterization of the nanoparticles

The preliminary characterization of the NPs was performed by means of electrophoretic light scattering (ELS), dynamic light scattering (DLS) and static light scattering (SLS).

2.3.1 Electrophoretic light scattering (ELS). The ELS measurements were performed in order to determine the average zeta potential ($\zeta$) of the nanoparticles using the Zetasizer NanoZS equipment (Malvern Instruments, UK). It measures the electrophoretic mobility ($U_e$) of the nanoparticles and converts the value to a $\zeta$ potential (mV) through the Henry’s equation:

$$U_e = \frac{2\varepsilon \zeta f(ka)}{3\eta}$$

where $\varepsilon$ is the dielectric constant of the medium and $\eta$ is the viscosity. The parameter $f(ka)$ is the Henry’s function, which has been calculated through the Smoluchowski approximation, $f(ka) = 1.5$ [14, 18]. Each $\zeta$ potential value reported herein is an average of 10 independent measurements with a repeatability of ±2%.

2.3.2 Dynamic light scattering (DLS). The DLS measurements were performed using an ALV/CGS-3 compact goniometer system consisting of a 22 mW HeNe linearly polarized laser operating at a wavelength of $\lambda = 633$ nm, an ALV 7004 digital correlator and a pair of avalanche photodiodes operating in the pseudo-cross-correlation mode. The samples were loaded in 10-mm-diameter glass cells and maintained at a constant temperature of $(25 \pm 1)$ °C. The autocorrelation functions reported herein are based on 3 independent runs with 10 s counting time. The data were collected and further averaged by using the ALV Correlator Control software, and they were analysed by using the Cumulant method according to Equation 2:
\[ \ln g_i(t) = \ln C - \Gamma t + \frac{\mu_2}{2} t^2 \ldots \] (2)

where the parameters \( \Gamma \) (relaxation frequency) and \( \mu_2 \) (second-order cumulant) could be determined. The hydrodynamic radius \( (R_H) \) of the nanoparticles was determined by using the Stokes-Einstein relation as:

\[ R_H = \frac{k_B T q^2}{6 \pi \eta \Gamma} \] (3)

where \( k_B \) is the Boltzmann constant, \( T \) is the absolute temperature, \( q \) is the scattering vector, \( \eta \) is the viscosity of the solvent and \( \Gamma \) is the mean relaxation frequency related to the diffusion of the nanoparticles. The polydispersity (PDI) of the samples was computed as \( \text{PDI} = \mu_2 \Gamma^{-2} \) [19, 20].

2.3.2 Static light scattering (SLS). The SLS measurements were also performed using the ALV/CGS-3 compact goniometer system. In the SLS mode, the scattering angle was varied from 30° to 150° with an angular step of 5°. At each angle, the light-scattered intensity was measured in triplicate and the average values were reported. The measured scattered intensity \( I(q) \) can be related to the radius of gyration \( (R_G) \) of the nanoparticles through the Guinier approximation:

\[ \ln I(q) = \ln I_{q \to 0} - \frac{q^2 R_G^2}{3} \] (4)

3. Results and discussion

Light scattering techniques were employed in order to analyze the stability of the promising bioactive compounds - LASSBio-1773 and LASSBio-1735 - after encapsulation into PLGA nanoparticles. Firstly, we carried out the zeta potential measurements. The magnitude of the zeta potential is an important physicochemical parameter that is related to the potential stability of nanoparticles. Large positive or negative zeta potential means that the particles tend to repel each other, preventing further aggregation/flocculation. Particles with zeta potentials more than ±30 mV are normally considered stable [18]. The zeta potential values determined for LASSBio-1773-loaded, LASSBio-1735-loaded and unloaded PLGA NPs were respectively -40 mV, -36 mV and -38 mV. These values shed some light on the stability of the formed NPs and we can also infer that these nanoparticles are stable and these results seem to indicate that the bioactive compounds were preferably encapsulated rather than adsorbed, as the zeta potential remains the same.

The DLS data were used to determine the mean hydrodynamic radius \( (R_H) \) and the polydispersity of the systems. Figure 4 displays the autocorrelation function for drug-free and drug loaded PLGA NPs. These curves suggest a monomodal distribution \( (\mu_2 \Gamma^{-2} \leq 0.16) \), which is related to the presence of polymeric nanoparticles and indicate the criteria for good quality (homogeneous) sample preparation. The mean values for both \( R_H \) and the PDI \( (\mu_2 \Gamma^{-2}) \) are given in Table 1.
Figure 4. Autocorrelation functions measured at a scattering angle of 90° for LASSBio-1773-loaded (red circles), LASSBio-1735-loaded (black squares) and drug-free PLGA (blue triangles) NPs.

The variation of the scattered intensity with the scattering angle was plotted to measure the mean static particle size ($R_G$). A decrease in $R_G$ as a function of the presence of both sulphonylhydrazone and N-acylhydrazone derivatives was observed.

Figure 5. Guinier plot – Angular variation of the scattering intensity as a function of $q^2$ for LASSBio-1735 and LASSBio-1773 compounds.
It is well established that the ratio $\rho = R_G/R_H$ is a characteristic parameter related to the conformation of polymer chains and self-assembled macromolecular objects in solution. The $\rho$-value of spherical objects is dependent on the inner structure and compactness, being close to 0.775 for very compact spheres, $\rho \sim 0.8$–0.9 for block copolymer micelles due to solvation phenomena and $\rho \sim 1.0$ for hollow spheres and vesicles [12, 13, 21-23]. The $R_G/R_H$ ratios (Table 1) suggest that these nanoparticles behave as compact spheres. The reduction of $R_G$, $R_H$ and $R_G/R_H$ as the probes are loaded is probably explained by strong hydrophobic interactions between PLGA and the bioactive compounds LASSBio-1773 and LASSBio-1735.

Table 1. Characteristics of the nanoparticles obtained by light scattering experiments. $R_H$ is the hydrodynamics radius, PDI indicates the polydispersity index ($\mu^2 / \Gamma - 2$), $\zeta$ is the zeta potential, $R_G$ is the radius of gyration and $R_H/R_G$ ratio indicates the conformation characteristic of the NPs.

<table>
<thead>
<tr>
<th>NPs</th>
<th>$R_H$ (nm)</th>
<th>PDI</th>
<th>$\zeta$ (mV)</th>
<th>$R_G$ (nm)</th>
<th>$R_G/R_H$</th>
</tr>
</thead>
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<tr>
<td>PLGA</td>
<td>132.5</td>
<td>0.03</td>
<td>-38(3)</td>
<td>103.4</td>
<td>0.78</td>
</tr>
<tr>
<td>LASSBio-1773</td>
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<td>0.16</td>
<td>-40(3)</td>
<td>97.9</td>
<td>0.76</td>
</tr>
<tr>
<td>LASSBio-1735</td>
<td>123.7</td>
<td>0.13</td>
<td>-36(3)</td>
<td>96.5</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Finally, it is important to highlight that further experiments are underway in order to determine the loading content and encapsulation efficiency of the developed nanosystems, which will then be followed by investigations regarding the release profile of the hydrophobic bioactive compounds.

4. Conclusions

In this work we demonstrated the feasibility of using PLGA to promote the encapsulation of new anti-diabetic sulphonylhydrazone and antitumor N-acylhydrazone derivatives. The produced polymeric nanoparticles were obtained by the nanoprecipitation method, which revealed to be effective in the encapsulation of these bioactive compounds. We also verified that both systems are highly hydrophobic. Our preliminary experimental data demonstrated the formation of nanosystems with promising features that should be further investigated in more details.

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