Colloids and Surfaces B: Biointerfaces 161 (2018) 670–676

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Full length article

Controlling insulin release from reverse hexagonal (H_{II}) liquid crystalline mesophase by enzymatic lipolysis

Tehila Mishraki-Berkowitz [a,1], Guy Cohen [b], Abraham Aserin [a], Nissim Garti [a,*]

[a] The Ratner Chair in Chemistry, Casali Institute of Applied Chemistry, The Institute of Chemistry, The Hebrew University of Jerusalem, Edmond J. Safra Campus, Jerusalem 9190401, Israel
[b] Skin Research Institute, Dead-Sea & Arava Science Center, Ein Gedi, Israel

ARTICLE INFO

Article history:
Received 27 June 2017
Received in revised form 29 October 2017
Accepted 11 November 2017
Available online 12 November 2017

Keywords:
Reverse hexagonal liquid crystal (H_{II} mesophase)
Thermomyces lanuginosa lipase (TLL)
Insulin
Drug release
Phosphatidylcholine (PC)
Monoolein

ABSTRACT

In the present study we aimed to control insulin release from the reverse hexagonal (H_{II}) mesophase using *Thermomyces lanuginosa* lipase (TLL) in the environment (outer TLL) or within the H_{II} cylinders (inner TLL). Two insulin-loaded systems differing by the presence (or absence) of phosphatidylcholine (PC) were examined.

In general, incorporation of PC into the H_{II} interface (without TLL) increased insulin release, as a more cooperative system was formed.

Addition of TLL to the systems' environments resulted in lipolysis of the H_{II} structure. In the absence of PC, the lipolysis was more dominant and led to a significant increase in insulin release (50% after 8 h). However, the presence of PC stabilized the interface, hindering the lipolysis, and therefore no impact on the release profile was detected during the first 8 h.

Entrapment of TLL within the H_{II} cylinders (with and without PC) drastically increased insulin release in both systems up to 100%. In the presence of PC insulin released faster and the structure was more stable.

Consequently, the presence of lipases (inner or outer) both enhanced the destruction of the carrier, and provided sustained release of the entrapped insulin.

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1. Introduction

Reverse hexagonal (H_{II}) lyotropic liquid crystals (LLCs) are self-assembled, long, and dense micelles, arranged in a two-dimensional array. Each of these cylinders is formed by a layer of surfactant molecules, in which the lipophilic moieties point outward and the hydrophilic heads point inward. The cylinders are filled with a hydrophilic solvent, which is usually water [1–3]. Hence, those structures enable the entrapment of lipophilic molecules between the lipid moieties, and water-soluble compounds within the aqueous domains. One of the most studied surfactants that create the LLCs' matrices and in particular the H_{II} mesophase is glycerol monooleate (GMO). This system was shown to have potential applications as a host system for drug delivery [4–7], food applications [8,9], and protein crystallization [5,10–14].

The H_{II} mesophase (containing GMO/water), that commonly assembled at 80 °C and inverts to other structures upon cooling [15], was stabilized at room temperature by the addition of a specific triacylglycerol ester (triglyceride, TAG) [16]. This stabilization enables utilization of the H_{II} system for incorporation of bioactive molecules, and in particular peptides and globular proteins [5,17]. It was shown that the H_{II} carrier improves the conformational stability of the model protein (lysozyme, LSZ) as a function of temperature, pH, and denaturing agent (urea) [18,19]. Furthermore, the H_{II} system enables sustained and controlled release [7,20–22].

Of special interest is the protein insulin, which is a hormone regulating carbohydrate and fat metabolism. Diabetes mellitus is a pathological condition characterized by an absolute or relative lack of insulin resulting in high blood glucose due to the inability to transport the glucose into cells (Type 1–juvenile, Type 2–adult) [23–25]. Hence, exogenous insulin must be administered by daily subcutaneous (s.c.) injection [26,27]. Although the oral route would be preferable, this has not been achievable due to denaturing of the insulin as it travels down the gastrointestinal tract (GIT). Therefore, we have recently focused our research on embedment of insulin into the H_{II} system [28–31], as it was shown that oral insulin can

* Corresponding author.
E-mail address: garti@mail.huji.ac.il (N. Garti).
[1] The results presented in this paper will appear in the Ph.D. dissertation of T.M.-B. in partial fulfillment of the requirements for the Ph.D. degree in Chemistry, The Hebrew University of Jerusalem, Israel.

https://doi.org/10.1016/j.colsurfb.2017.11.031
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be comparable to s.c. insulin with regard to glycemic efficacy and safety [32,33].

Insulin-loading into the H_{II} carrier was found to destabilize the structure [29] and to disorder the interface [28], as insulin was located in the water core and in between the GMO headgroups. Insulin encapsulation enhanced the formation of the α-helix structure, which characterizes a more stable conformation, compared to when it is dissolved in aqueous solution [30]. Addition of the co-surfactant phosphatidylcholine (PC) to the H_{II} system significantly increased insulin's cumulative release [31]. However, a major part of the insulin content still remained "trapped" in the H_{II} matrix. Rupture of the hexagonal structure would enable the release of the "trapped" insulin and control the insulin release rate.

Thermomyces lanuginosa lipase (TLL) was shown to cause lipolysis of the LLCs' structures and to provide structural transitions, as the lipid composition was modified during lipolytic processes [34–36]. Our laboratory's previous study demonstrated that addition of TLL to the H_{II} system disordered and decomposed the hexagonal mesophase, and thereby enhanced the diffusion of the encapsulated drug (sodium diclofenac) [37]. Thus, addition of TLL into the aqueous core of the insulin-loaded H_{II} mesophase could enable control of the insulin release rate. This would assist in the development of a novel insulin-carrier that could fully digest and release all the entrapped insulin. Additionally, since lipases perform an essential role in digestion by the GIT, examination of the insulin-loaded H_{II} system in the presence of TLL can emulate the GIT.

In the present study we aimed to design a smart biological carrier for insulin that would enable an efficient and sustained and controlled delivery. Insulin release would be examined in two aspects: (1) upon addition of TLL into the aqueous environment, and (2) upon addition of TLL directly into the insulin-loaded H_{II} mesophase. Additionally, the relationship between insulin release and the breakage of H_{II} structure was explored.

2. Experimental

2.1. Materials

Monoolein, distilled glycerol monooleate (GMO) (min. 97 wt% monoglycerides, 2.5 wt% diglycerides, and 0.4 wt% free glycerol; acid value 1.2, iodine value 68.0, and melting point 37.5 °C) was obtained from Riken Vitamin Co. (Tokyo, Japan). Phosphatidylcholine (PC) of soybean origin (Epikuron 200, min. 92 wt% PC) was purchased from Degussa (Hamburg, Germany). Tricaprylin (triacylglycerol, TAG) (97–98 wt%), *Thermomyces lanuginosa* lipase (TLL; 100,000 U/g), phosphate buffered saline (PBS; 0.01 M; pH 7.4), and dichloromethane were purchased from Sigma Chemical Co. (St. Louis, MO, USA). NovoRapid (insulin aspart; 100 U/mL solution for injection) was purchased from Novo Nordisk (Bagsvaerd, Denmark). The water was double distilled. All components were used without further purification.

2.2. Preparation of H_{II} mesophases

Four reverse hexagonal liquid crystal systems composed of GMO/TAG/(insulin solution), GMO/PC/TAG/(insulin solution), GMO/TAG/(insulin solution)/TLL, and GMO/PC/TAG/(insulin solution)/TLL with weight ratios of 72/8/20, 63/10/7/20, 71.1/7.9/20/1, and 62.1/10/6.9/20/1, respectively, were prepared. In order to study the influence of PC and TLL on the mesophases, they were solubilized at constant concentrations while decreasing the GMO and TAG concentrations but keeping the weight ratio of GMO/TAG (9:1) and insulin solution (NovoRapid) content of 20 wt% constant. The weighed quantities of GMO, TAG, and PC were mixed while heating to 80 °C. The samples were stirred and cooled to 32 °C. An appropriate quantity of the insulin solution and TLL were added and the samples were stirred. Additionally, 1000 U/g TLL solution in PBS was prepared.

2.3. Insulin release kinetics

A vertical Franz diffusion cell (PermeGear, Inc., Hellertown, PA, USA) was used to determine the insulin release profile of the various formulations. The receptor chamber was filled with 5 mL PBS (pH 7.4) with or without 1000 U/g TLL. Then, freshly prepared liquid crystal formulations were mounted on a supporting membrane (100 μm; Millipore, Bedford, MA, USA) and clamped over the filled receptor (diffusion area 0.64 cm^2). The apparatus was constantly stirred (500 rpm) and kept at 32 ± 0.5 °C. At 0.5, 1, 3, 5, and 8 h, 0.4 mL samples were taken from the receptor chamber, and insulin content was measured twice by enzyme-linked immunosorbent assay (ELISA) (Mercodia, Sweden). Each sampling was followed by insertion of 0.4 mL of fresh PBS or 1000 U/g TLL solution in PBS. The experiment was repeated three times.

2.4. Cross-polarized light microscope

Microscopic observations were performed using a Nikon model Eclipse 80i microscope equipped with Digital camera model DXM 1200C (Nikon, Tokyo, Japan). The samples were inserted between two glass microscope slides and observed with cross-polarizers.

2.5. Lipolysis kinetics

Lipolysis of the H_{II} mesophases was monitored by breakage of the H_{II} structures at various time points. Fifty mg of the appropriate insulin-loaded H_{II} system were inserted in a test tube with 0.2 mL PBS with or without 1000 U/g TLL, for different time periods. 5 mL of dichloromethane were added to each test tube, and stirred to complete breakage of the H_{II} structure and phase separation. A sample was then immediately collected from the dichloromethane phase (to avoid further lipolysis). The samples were injected into the GCMS for analysis of the lipid composition.

2.6. Gas chromatography–mass spectroscopy (GC–MS)

Chromatographic measurements were performed using GC–MS with 7890A GC and 5975C MSD detector (Agilent Technologies, Santa Clara, CA, USA). The ionization source was of an electron ionization (EI) mode. Solution was injected into a DB-5MS capillary fused silica column (0.25 mm diameter, 15 m length, 0.25 μm internal coating) (Agilent Technologies). Injector temperature was set to 290 °C. The column temperature was measured from 60 °C to 290 °C at 25 °C/min. Regression and statistical analysis of the obtained data were conducted using the GC–MS ChemStation software package and NIST library.

3. Results

3.1. Enzymatic lipolysis of the insulin-loaded H_{II} mesophase

The stability of the insulin-loaded H_{II} structures, with and without PC, was studied by addition of TLL to the environment of the H_{II} systems or into the core of the H_{II} structures. The TLL that was placed in the aqueous environment is mimicking the situation in our guts. We termed this TLL, in short, the "outer TLL". While by adding the TLL in the aqueous channels together with the insulin its effect was more localized and we hoped to obtain better control of insulin release. We termed this TLL, in short the "inner TLL". When

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