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Original article

A second-generation micro/nano capsules of an endogenous primary un-metabolised bile acid, stabilized by Eudragit-alginate complex with antioxidant compounds

Armin Mooranian^a, Nassim Zamani^a, Momir Mikov^b, Svetlana Goločorbin-Kon^c, Goran Stojanovic^d, Frank Arfuso^e, Bozica Kovacevic^a, Hani Al-Salami^{a,*}

^a Biotechnology and Drug Development Research Laboratory, School of Pharmacy and Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, Perth, Western Australia, Australia

^b Department of Pharmacology, Toxicology and Clinical Pharmacology, Faculty of Medicine, University of Novi Sad, Novi Sad, Serbia

^c Department of Pharmacy, University of Novi Sad, Novi Sad, Serbia

^d Faculty of Technical Sciences, University of Novi Sad, Novi Sad, Serbia

^e Stem Cell and Cancer Biology Laboratory, School of Pharmacy and Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, Perth, Western Australia, Australia

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ABSTRACT

Bile acids (BAs) are amphiphilic compounds and of recently have demonstrated wide range of formulation stabilizing effects. A recent study showed that primary un-metabolised bile acids (PUBAs) have β -cell protective effects, and synergistic antidiabetic effects when combined with antioxidant and anti-inflammatory drugs, such as probuocol (PB). Thus, this study aimed to design and test microcapsules containing a PUBA incorporated with PB and an alginate-Eudragit matrix.

Six types of microcapsules were developed without (control) or with (test) PUBA, and tested for internal and external features and β -cell protective effects.

The incorporation of PB-alginate-Eudragit with PUBA produced stable microcapsules but did not exert consistent positive effects on cell viability in the hyperglycaemic state, which suggests that PUBA in alginate-Eudragit matrices did not exhibit synergistic effects with PB nor exerted antidiabetic effects.

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1. Introduction

Bile is a complex fluid stored in the bladder and contains water, electrolytes, and organic molecules including bile acids, bile salts, cholesterol, phospholipids and bilirubin. Bile is normally flows from the bile duct into the small intestine as a response to food

* Corresponding author at: Senior Lecturer of Pharmaceutics Founder of Biotechnology and Drug Development Research Laboratory School of Pharmacy and Biomedical Sciences Curtin Health Innovation Research Institute (CHIRI) Curtin University, Perth, WA, Australia.

E-mail address: hani.al-salami@curtin.edu.au (H. Al-Salami).

URL: <http://healthsciences.curtin.edu.au/schools-and-departments/biomedical-sciences/research/biotechnology-and-drug-development-research-laboratory/> (H. Al-Salami).

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ingestion (Houten et al., 2006a). The main endogenous bile acids are primary (cholic and chenodeoxycholic acids) and secondary (deoxycholic and lithocholic acids) bile acids. Bile acids (BAs) are a group of structurally diverse molecules that share similar steroid-backbone structure and primarily synthesized in the liver from cholesterol catabolism. They are the main components of bile and have amphiphilic chemical structure with surfactant-like properties. BAs can regulate their own enterohepatic circulation and metabolic processes, and exert regulatory effects on homeostasis of triglyceride, cholesterol, energy, and glucose balance (Houten et al., 2006b). Primary bile acids are synthesized in liver cells from endogenous or dietary cholesterol and conjugated to glycine or taurine to form primary conjugated bile acids. Starting from the duodenum which is the upper part of the small intestine, the conjugated bile acids are metabolised by the gut microbiota into secondary bile acids before being reabsorbed back into the liver and metabolised again, in the process of enterohepatic recirculation (Ridlon et al., 2006). Approximately 95% of bile acids secreted into the gut is reabsorbed from the intestine back into the circulation

via bile acid transporters, while about 600 mg/day of bile acids is excreted from the body in the faeces (Roberts et al., 2002).

Recent studies in our laboratory have expanded the roles of bile acids to include microcapsule-stabilizing effects for the oral delivery of hydrophobic drugs and also permeation-enhancing effects for optimising bioavailability of poorly absorbed drugs, in diabetes (Al-Salami et al., 2008; Mikov and Golocorbin-Kon, 2012; Lalic-Popovic et al., 2013; Mooranian et al., 2014a; Mooranian et al., 2014b; Mooranian et al., 2015a; Mooranian et al., 2015b; Mooranian et al., 2015c; Mooranian et al., 2015d; Lalic-Popovic et al., 2016; Mathavan et al., 2016; Mooranian et al., 2016a; Mooranian et al., 2016b; Mooranian et al., 2016c; Mooranian et al., 2016d; Mooranian et al., 2016f; Mooranian et al., 2016g; Golocorbin-Kon et al., 2017; Mathavan et al., 2017; Mooranian et al., 2018a; Mooranian et al., 2018c; Mooranian et al., 2018d; Mooranian et al., 2018e; Mooranian et al., 2018f; Mamo et al., 2018a; Mamo et al., 2018b; Takechi et al., 2017; Mamo et al., 2017). The primary un-metabolised bile acid cholic acid (CA) has shown unique properties compared with other bile acids including its ability to exert positive effects on release profile of the hydrophobic antidiabetic drug gliclazide (G), when microcapsulated using sodium alginate (SA) matrix (Mooranian et al., 2015d). CA had similar effects on the antioxidant antilipidemic drug, probucol (PB) (Mooranian et al., 2016e). However, the release profiles of G and PB did not maintain constant release rate in CA and SA mixture and the targeted delivery profiles of the drugs within the microcapsules were not highly selective for gut release, nor efficiently optimised, and thus delivery and absorption were not adequately efficient.

PB has shown great potential in the treatment of diabetes mellitus through its antioxidant and antiinflammatory effects and to date, its side effects remain significant due to poor targeted delivery and absorption profiles (Jeon et al., 2011; Komura et al., 1997). Accordingly, a new smart polymer needs to be incorporated into the SA-CA matrix and produce new microcapsules that may have better targeted-delivery and better release profiles of PB. Eudragit (ED) is a smart polymer which has shown potential applications in targeted drug delivery and release of orally administered antidiabetic drugs (Barakat et al., 2013). Its incorporation with SA-CA microcapsules have not been tested, and thus, it is one of the main aims of this study. Accordingly, this study aimed to fabricate new microcapsules using SA and ED polymers, and test how CA incorporation will optimise the PB targeted release and cellular absorption, using SA-ED microcapsules.

2. Materials and methods

2.1. Materials and cell culture

Cholic acid, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, sodium alginate, and probucol were from Sigma-Aldrich Corporation (Australia). Ultrasonic gel was purchased from Scharlab S.L, Australia. Eudragit polymers RL30D, NM30D and RS30D were from Evonik Industries (Victoria, Australia), 2',7'-dichlorofluorescein diacetate (DCFH-DA) was from Sigma-Aldrich Corporation (Australia), and 2,2'-azobis-2-methyl-propanimidamide, dihydrochloride (AAPH) was from Sapphire Bioscience (Australia).

Pancreatic β cell line, NIT-1, were grown using optimized media consisting of Dulbecco's Modified Eagle's medium (DMEM) and free amino acids, as per our published methods (Mooranian et al., 2017d).

Six different formulations were prepared, three of control and three of test. The control formulations consisted of sodium alginate (2%) with one of the following Eudragit polymers: NM30D (F1), RL30D (F3) and RS30D (F5), while the test had the same formulations but with cholic acid (4%) added (F2, F4 and F6 respectively).

Microcapsules were formed using the six microencapsulating formulations (Mooranian et al., 2014a; Mooranian et al., 2015b; Mooranian et al., 2014d). Microencapsulation was done using the Büchi B-390 encapsulator (Büchi, Switzerland), with vibrational nozzle system in order to generate droplets of suspended PB within the polymer matrix, while the final microcapsules were formed in a 2.5% w/v calcium chloride ionic gelation hardening bath with magnetic stirring for two minutes initially with intermittent mixing over 15 min total based on established methods (Mooranian et al., 2014d; Mooranian et al., 2018b).

2.2. Morphology, size distribution, Zeta-potential, thermal and stability index and water uptake property measurements

Morphology and size distribution of microcapsules were determined using Zeiss Neon 40EsB (FIBSEM, USA) and Mastersizer 2000 (Malvern, UK), and the electrokinetic (Zeta) potential of the formulations were determined using Malvern Zetasizer 3000HSa (Malvern, UK), as per our well-established methods (Mooranian et al., 2014b; Mooranian et al., 2014c). Thermal profile, stability index, and water uptake profile of microcapsules were determined as following: (1) Thermal profile was assessed using differential scanning calorimetric assays via DSC 6000 instrumentation (PerkinElmer, Australia), where 5 mg samples of microcapsules were heated in a temperature range of 20–300 °C, (2) Stability index was assessed by exposing the microcapsules to shaking and vibration for 14 days and count intact microcapsules as a percentage of total microcapsules, and (3) Water uptake was assessed by weighing freshly dried microcapsules immediately after fabrication and 14 days later to measure moisture gain. Microcapsules weight and numbers were standardized for all experiments, using our well-established methods (Mooranian et al., 2014a; Mooranian et al., 2014d; Mooranian et al., 2014e).

2.3. Drug release, cellular oxidative stress assay and survival measurements

Probuco release at 7.4pH was measured using our in-built system (Fig. 1) based on UV-Vis dissolution machine (PerkinElmer Lambda 25; PerkinElmer, USA) integrated with a closed-up loop system connected to a Masterflex C/L Variable-Speed Tubing peristaltic pump (Masterflex, USA), as per our well-established methods (Mooranian et al., 2015f; Negrulj et al., 2016).

Cell viability was measured using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; MTT) at two levels of glucose concentrations, 6.3 and 38.4 mM as per our well-established methods (Mooranian et al., 2017b; Mooranian et al., 2017c). Effects of microcapsules on cell survival and viability, and antioxidants activities of the microencapsulated probucol with or without the un-metabolised cholic acid were measured at the end of two days. Antioxidant activities were measured using oxidative stress testing at hyperglycaemic state with a mixture of dichloro-dihydrofluorescein diacetate (4.6 mM) and 2,2'-azobis-2-methyl-propanimidamide, dihydrochloride (18.44 mM), in which cells were incubated and fluorescence measurements was carried out using a plate reader (Enspire, PerkinElmer, USA), which is used as a measure of oxidized radical species concentrations.

2.4. Statistical analysis

Data are stated as means \pm SEM from triplicate analyses of the same batch of microcapsules and statistical analysis was performed using parametric/non-parametric or one-way ANOVA followed by Tukey posthoc as appropriate. GraphPad Prism Version X8.2 was used (GraphPad, USA) and p-value < 0.05 was considered significant.

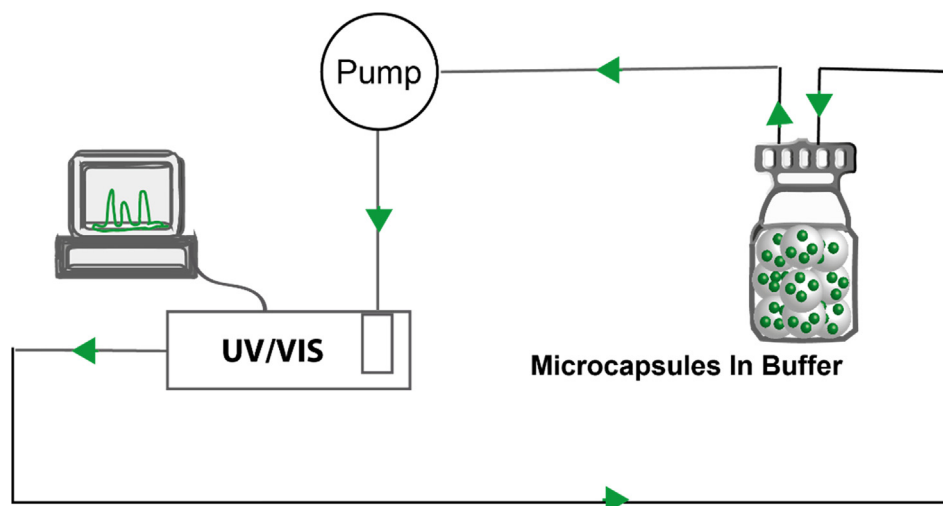


Fig. 1. UV-Vis dissolution system.

3. Results and discussion

3.1. Microcapsule surface characteristics and physico-chemical stability measurements

Size and shape of microcapsules remained similar independent of microcapsules' ingredients or cholic acid incorporation, which is consistent with our previously published studies on alginate-based microcapsules (Mooranian et al., 2014d; Mooranian et al., 2015d). This suggests that the ionic gelation vibrational jet-flow (IGVJF) method used is robust and efficient (Mooranian et al., 2017a). Surface analysis of microcapsules showed solid non-porous membrane, regardless of microcapsules' ingredients. The lack of porosity may optimise the controlled release profile of probucol from the microcapsules. Effect of membrane porosity on drug release has been investigated in the literature. Donbrow M, *et al*; investigated the impact of Eudragit polymer mixture (RL and RS) on microcapsule' wall porosity, and effects of external media on controlling release of a drug from microcapsules. Using theophylline, potassium dichromate or sodium chloride as model core materials the authors found that release rates increased with polar group content of the mixtures, and release was accelerated when the external medium contained sodium lauryl sulphate as a wetting agent. The release rate was sensitive to agitation intensity only at low wall to core ratios, and buffer ions penetrated coatings readily, changing theophylline release rates and providing clear evidence of diffusion via a pore-capillary mechanism. This illustrates the importance of types of Eudragit polymer used and porosity and size of microcapsules, on drug release profile and potential biological effects (Donbrow et al., 1995).

Electrokinetic stability (Zeta-potential) was increased ($p < 0.01$) in all microcapsules, due to cholic acid incorporation which suggests improved stability (Duro et al., 1998; Mirhosseini et al., 2007). Thermal capacity and physical stability of microcapsules remained consistent upon cholic acid incorporation, which suggests that the formed suspension was stable with or without cholic acid incorporation. This also suggests that thermal capacity and stability of microcapsules were similar among all formulations and were not significantly influenced by type of Eudragit polymer or presence of cholic acid. The decrease in water index is consistent with electrokinetic stabilization effects of cholic acid, and suggests stronger microcapsules' membranes with improved ability of matrix to withhold water contents and resist dehydration (Mooranian et al., 2014d; Mooranian et al., 2014e). The increase

in electrokinetic and water index stability by cholic acid incorporation were consistent among all formulations, and suggest stable microcapsules and improved physico-chemical properties. This may result in improved probucol release patterns and better biological effects (Fig. 3).

3.2. ProbucoL pH-release, cellular oxidative stress assay and survival measurements

Drug release patterns were consistent regardless of formulation ingredients or cholic acid incorporation, which suggest that microcapsules' matrix was uniform and probucol distribution was even within the layers of all microcapsules. This also suggests that microcapsules' disintegration and drug dissolution processes were consistent and not affected by variation in microcapsules' ingredients or cholic acid incorporation. The consistent release patterns of probucol were uniphasic, which is an improvement, compared with our previous studies where release patterns were diphasic or multiphasic (Mooranian et al., 2015e; Negruj et al., 2016; Mooranian et al., 2015f). CA was considered as an excipient hence, measuring its release was not one of the aims of this study, although PB release suggest that CA would have similar release patterns as part of the microcapsules' disintegration and release profiles. Improved drug release via Eudragit incorporation has been published in the literature. Wulff R, and Leopold CS, investigated release patterns of theophylline pellets coated with blends of Eudragit polymers. The authors have shown that theophylline release was dependent on pH of media, and was mainly either affected by coating materials or ratio of the blends of Eudragit polymers, and these blends offer great promise for improved drug release properties (Wulff and Leopold, 2014). Moustafine et al. investigated the interplay between different Eudragit polymers, and how this modifies drug release. The authors explored interpolymer interactions between countercharged RL30D and FS30D and their effects on release of the drug, diclofenac, after oral administration. They found that the interaction between countercharged copolymers during passage in gastrointestinal tract can strongly modify the release profile of diclofenac, and can improve its controlled release, after an oral dose (Moustafine et al., 2012).

Microcapsules' effects on oxidative stress levels showed significant antioxidant activities only by NM30D microcapsules after cholic acid incorporation, and these positive effects were consistent with effects on cell survival (Fig. 2). This suggests a correlation between antioxidant effects and cell viability and functions, at the

hyperglycaemic state, and these effects are formulation-dependent as they were not observed by RL30D and RS30D microcapsules without or with addition of cholic acid. The effects of Eudragit polymers on cell biology have been explored in the literature. Pandey et al. investigated using Eudragit S100 in nanoparticles for the sustained release delivery of the anticancer drug, capecitabine. The authors have shown that the nanoparticles were taken intracellularly by HT 29 adenocarcinoma cells, without causing significant cytotoxic or apoptotic effects of the Eudragit polymers at normoglycemic state, which is consistent with results in Fig. 2 (Pandey et al., 2016). Moreover, Abdel-Wahhab et al. investigated the cellular uptake of Eudragit RL nanoparticles by THP-1 human monocytic cells, and the nanoparticles' hematological and erythrocytic effects, in rats. The authors have shown that the nanoparticles caused significant hematological disturbances in platelets, red

blood cell and white blood cells, but were comparable with control, which suggests variable biological effects of Eudragit polymers depending on formulation and the delivery systems deployed (Abdel-Wahhab et al., 2014). This is consistent with our findings (Fig. 2) showing similar cell survival rate at normoglycemic state, regardless of formulation used, but reduction in cell survival at the hyperglycaemic state, due to potential glucotoxicity, which was ameliorated by NM30D-cholic acid microcapsules (Fig. 3).

Accordingly, results of this study illustrate the effects of co-encapsulation of three Eudragit polymers (NM, RL and RS – 30D) on delivery of the drug probucol, without or with the un-metabolised bile acid cholic acid. Results show formation of regular oval microcapsules with improved electrokinetic, thermal and physical stability being brought about by incorporation of the un-metabolised primary bile acid (Fig. 2). Results also show that

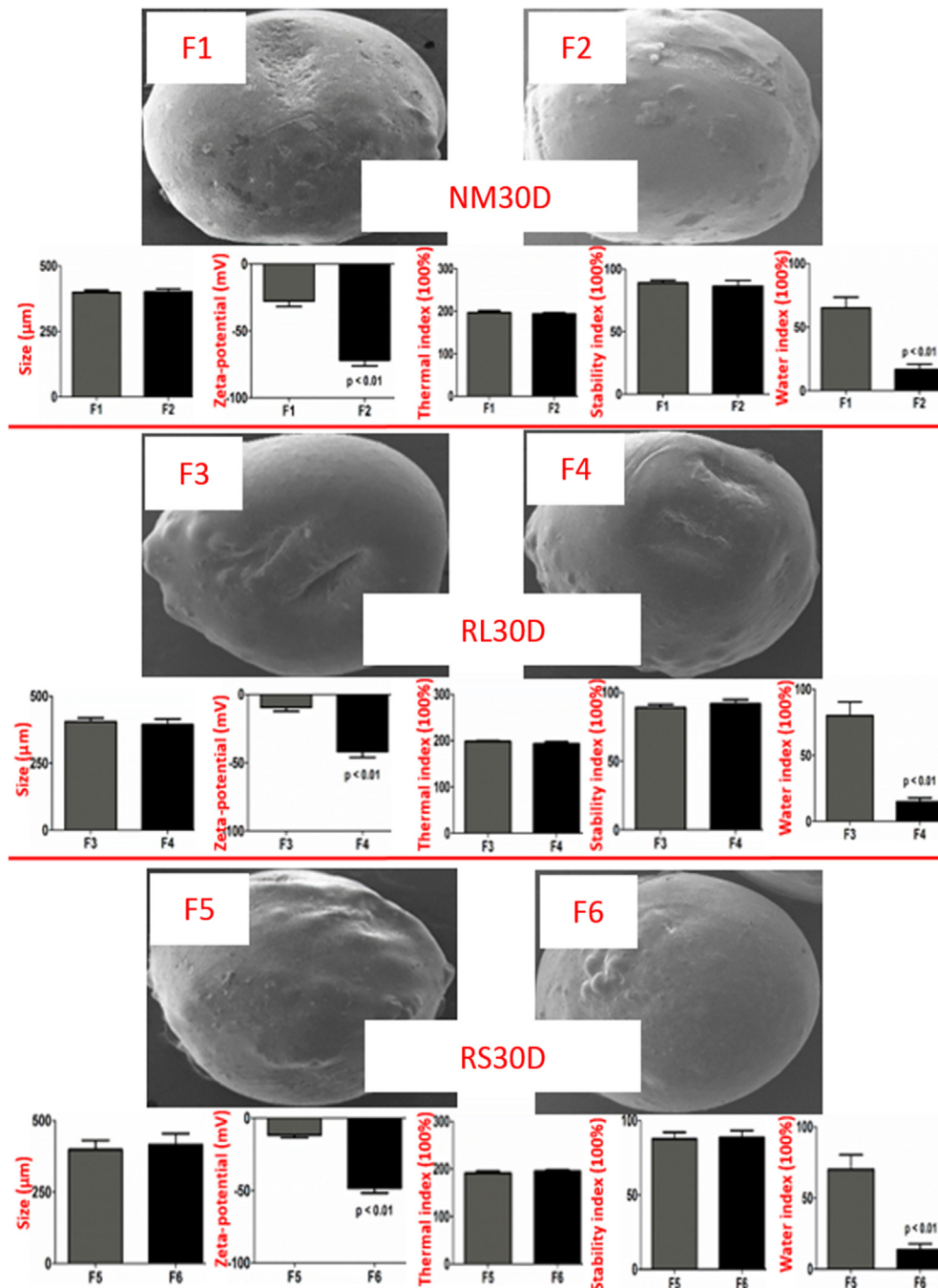


Fig. 2. Surface features, and physicochemical characteristics of F1-F6. Data are mean \pm standard error of the mean, n = 3.

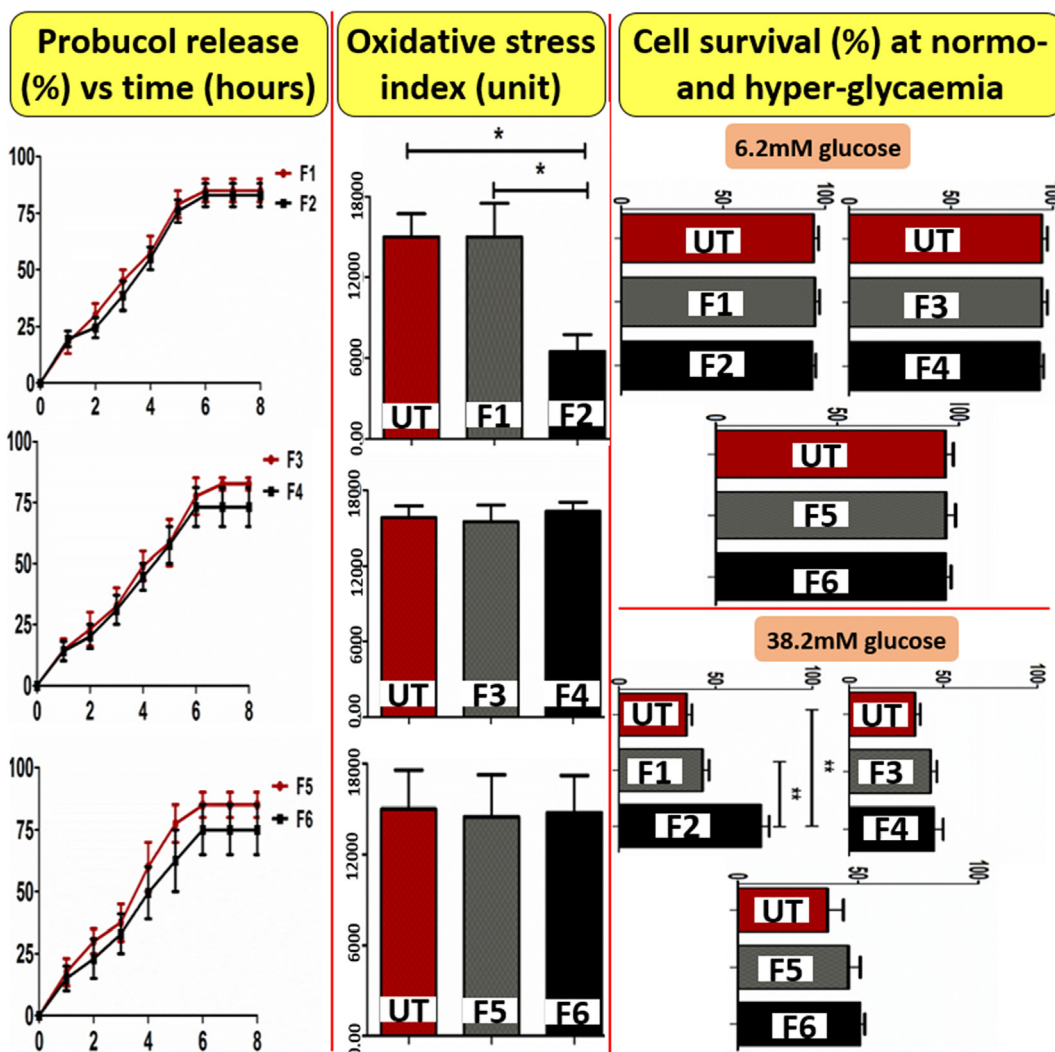


Fig. 3. Release profile at pH 7.4, and biological effects of control (F1, F3 and F5) and test (F2, F4 and F6) groups, and UT is untreated. Data are mean \pm standard error of the mean, $n = 3$.

probucol release patterns are improved by Eudragit incorporation, and oxidative stress in β -cells was minimized and cell viability was maximized by NM30D-cholic acid incorporation into the probucol microcapsules, which suggests potential beneficial biological effects of the microcapsules on probucol delivery and biological effects, on β -cells, *in vivo*. The results are limited by the fact that one bile acid concentration and one cell type were examined rather than all bile acids (there are >100 types) over wide range of concentrations (e.g. 5 concentrations per bile acid) in multiple cell types (e.g. 5 or 6). Future studies need to explore oral applications of the NM30D-cholic acid microcapsules in probucol delivery and applications in diseases, such as diabetes mellitus.

Declaration of Competing Interest

Al-Salami H has been and is currently receiving funding from Beijing Nat-Med Biotechnology Co. Ltd.

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