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Invited Review

Multilayer nanoencapsulation: A nanomedicine technology for diabetes research and management

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ABSTRACT

Nanothickness encapsulation using a layer-by-layer technique has applications in several areas of diabetes research, including improved glucose sensors, islet cell transplantation and oral insulin delivery. We have fabricated microvesicles containing a fluorescence lifetime-based glucose sensing system, with bacterial glucose-binding protein as the glucose receptor. Such sensors are suitable for impregnation in the dermis as a 'smart tattoo' type of non-invasive glucose monitoring technology. Nanoencapsulation of islet cells is intended to alleviate the immediate blood-mediated inflammatory reaction which is responsible for early islet loss post-transplant. In an allogeneic diabetic mouse model, nanoencapsulated islets with phosporylcholine-modified polysaccharide coating, significantly extended survival of transplanted islets. In early studies aimed at formulating an effective oral insulin preparation, insulin-chitosan colloids coated with nanolayers of chitosan and heparin had enhanced acid stability and effectively lowered blood glucose in an animal model.

Contents

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1. Introduction

Nanomedicine involves the manufacture, measurement and clinical application of very small (nano)-scale structures (usually 1–100 nm in size), – membranes and films, particles, vesicles, probes and sensors being typical examples [1,2]. Such structures usually have altered properties, such as increased strength, or changed activity or porosity, compared to larger-scale constructions. This article reviews one of the most active and promising research areas in nanomedicine: the use of multilayered nano-thickness films for encapsulating cells, sensors and drug delivery systems. The special characteristics of these films include rapid transit of some substances but isolation of others, control of biocompatibility and the local in vivo environment, and robust containment. Applications of this technology are being seen in at least three major areas of diabetes: in vivo glucose sensing, cell therapeutics (particularly islet cell transplantation) and insulin delivery (potentially for oral administration).

2. Layer-by-layer technology

The layer-by-layer (LBL) technique for formation of nanomembranes involves the alternate deposition of polymer electrolytes of positive or negative charge on a template [3,4]. Examples of some of the polymers used are polyamino acids (e.g. poly[L-glutamic acid] and poly[L-lysine]), polysaccharides (e.g. heparin, alginate and chitosan) and synthetic polymers (e.g. poly[allylamine hydrochloride] and poly[styrene sulphate]). Originally developed as a coating technology for flat surfaces, LBL technology has been now extensively used for encapsulation of particles, proteins and cells. The amount deposited in each layer is self limited, and independent of time and concentration. The mild nature of the coating procedure and the ability to tune permeability and biocompatibility by the number of layers, the composition of the polymers and the coating conditions, and the ability to incorporate additional bioactive molecules in the layers, have made LBL technologies particularly suitable for encapsulation and in vivo administration of biological moieties such as proteins and cells.

3. Nanoencapsulation for improved glucose sensors

3.1. *Fluorescence-based sensors and 'smart tattoos'*

Non-invasive glucose sensing has long been a dream of patients with diabetes and researchers, and many technologies have been investigated, including near-infrared (NIR), Raman, photoacoustic and impedance spectroscopy, optical coherence tomography, polarimetry and reverse iontophoresis [5]. None has yet seen application in clinical practice, largely because of interferences and imprecision and alternative technologies are therefore being explored. In recent years, fluorescence has emerged as a promising technology for both minimally invasive, re-implantable glucose sensors and non-invasive glucose sensing [6]. Fluorescence is very sensitive and not subject to electroactive interferents in the tissues, and since the tissues are transparent to several centimetres of NIR light, when the fluorophore is excited and emits in the NIR range, intradermally or subcutaneously impregnated fluorescence-based sensors can be potentially used as a non-invasive technology [2], with measurement from the skin surface. This so called 'smart tattoo' concept [2] was probably first suggested by Russell et al. [7], though in the context of hydrogel glucose-sensing microspheres, rather than encapsulated sensors. Recent work on hydrogel fibres incorporating a fluorescent glucose sensing system with excitation at 350–420 nm and emission at 460–520 nm, implanted into the dermis of the mouse ear and with skin-surface fluorescence recording [8], suggests that at least under some circumstances (perhaps if the sensor is close to the skin surface) the fluorophore need always not be operative in the NIR range.

Florescence changes can be measured as either intensity or the lifetime of the decay [6,9]. Since the latter is essentially independent of light scattering, fluorophore concentration and photobleaching, it is particularly useful for in vivo sensors such as a smart tattoo. Thus, although implanted fluorescence probes may become coated in vivo by protein or encapsulated by cells which would decrease fluorescence intensity, the lifetime will remain relatively constant.

3.2. *Nanoencapsulated glucose sensors*

Microvesicles created by LBL encapsulation and containing fluorescence-based glucose sensors have been described by our own group [2,10], as well as McShane et al. [11–15]. In general, hollow-vesicle sensors can be formed by LBL deposition around a removable template such as calcium carbonate which can then dissolved (e.g. using ethylenediaminetetraacetic acid, EDTA) [15–17]. One way of introducing the sensing element into the vesicles is by uptake into preformed vesicles (loading by diffusion), with alteration of the membrane pore size by changing factors such as pH or ionic strength, so as to either increase uptake during loading or trap the sensing material after uptake [15]. A method of higher efficiency is first to adsorb or absorb the sensing element to a sacrificial template (or to co-precipitate the template and sensing reagent), form LBL nanomembranes around the template/ sensor and then dissolve the template [10,16,17] (Fig. 1).

Several fluorescence-based glucose sensing assays have been incorporated into multilayer nanocapsules, operating as either competitive or non-competitive systems. In competitive systems, there is a change in fluorescence resonance energy transfer (FRET) when glucose binds to a fluorescent-labelled receptor such as tetramethyl rhodamine isothiocyanate labelled concanavalin A (FRET acceptor), in competition with a fluorescent glucose analogue such as fluorescein isothiocyanate-labelled dextran (FRET donor) [12]. Other competitive systems use apo glucose oxidase (glucose oxidase with the prosthetic group removed) as the glucose receptor, labelled with a variety of acceptors, together with dextran labelled with one of several donors [13,14]. Entrapped glucose oxidase may also be used as a sensor with detection of oxygen consumption via a co-entrapped oxygen-sensitive dye Ru(dpp) [18].

3.3. LBL nanoencapsulated glucose sensor based on glucose-binding protein

Most of our recent sensing research has been centred on fluorescently-labelled bacterial glucose/galactose-binding protein (GBP) as the glucose receptor, configured in a non-competitive assay system. When glucose binds to GBP, the tertiary structure of the protein changes markedly, with the two lobes of the molecule closing round glucose and the binding site [19]. We covalently linked the environmentally-sensitive fluorophore, badan (the fluorescence of which increases in a non-polar environment), to a cysteine mutation near the binding site of GBP, so that the consequent decrease in polarity at the binding site is marked by an increase in fluorescence intensity and mean lifetime [10,20].

When measuring fluorescence lifetime by time-correlated single-photon counting, the fluorescence decay can be resolved into two components: a short-lifetime component of 0.8 ns, equivalent to the open form of GBP with no glucose bound and badan in a polar environment, and a long-lifetime component of 3.1 ns equivalent to the closed form of GBP with bound glucose and badan in a hydrophobic environment [10]. When glucose is added to GBP–badan, the fraction of the long-lifetime component increases and the fraction of the short lifetime component decreases [10].

Native GBP has a binding constant (K_d) in the micromolar range and therefore is unsuitable for use as a clinical sensor, so we engineered a series of mutants of GBP with the intention of increasing the K_d. We found that the triple mutant H152C/ A213R/L238S has a K_d of 11 mmol/l and an operating range from 1–100 mmol/l glucose, and is therefore suitable for use in a clinical sensor [21].

We constructed microvesicles encapsulating the GBP–badan and suitable for smart tattoo, by first absorbing GBP–badan onto a template of calcium carbonate, then forming the shell around the template using the LBL technique (alternating poly[L-lysine] with poly[L-glutamic acid] or heparin), followed by dissolution of the template with EDTA (Fig. 1). The changes in the fluorescence lifetime on addition of glucose can be visualised by fluorescence lifetime imaging microscopy [10] (Fig. 1).

The challenges for the future with a smart tattoo include maintaining survival of the microsensors in vivo, showing lack of toxicity of the materials and efficient fluorescence recording

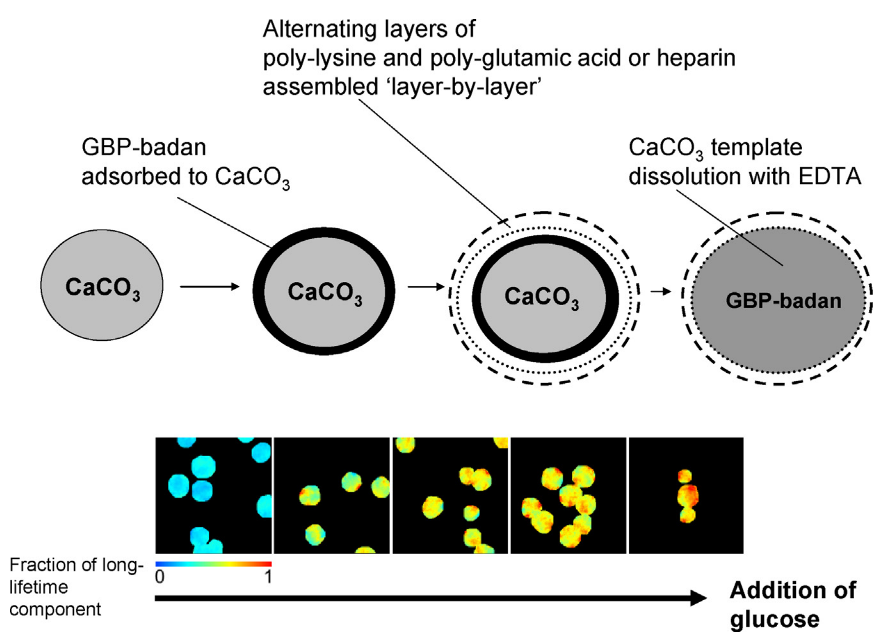

Fig. 1 – Upper: encapsulation of glucose-binding protein (GBP) labelled with the fluorophore, badan, in nanoencapasulated vesicles. GBP-badan is absorbed to calcium carbonate microparticles, then multiple polymer nanolayers are deposited, followed by dissolution of the template with EDTA. Lower: addition of glucose to nanoencapsulated glucose-sensing vesicles increases the fluorescence lifetime, and can be visualised by fluorescence lifetime imaging microscopy (blue = low fraction and red = high fraction of long-lifetime, closed form of GBP). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

from the skin surface. Though, as mentioned above, there is some evidence that fluorophores that are excited and emit in the same spectral region as badan can be interrogated from the skin surface when impregnated into the dermis [8], better functioning may be achieved by substituting badan for an environmentally sensitive fluorophore operative in the NIR region.

4. Nanoencapsulation of transplanted pancreatic islets

4.1. *The advantages of LBL encapsulation*

In addition to an insufficient supply of donor islets and immune rejection unless toxic immunosuppressant drugs are used, islet transplantation as a viable routine therapy for type 1 diabetes is currently also limited by early loss of functional islets after the clinically preferred intrahepatic transplantation, attributed to a deleterious response by the host innate immune system, and known as an immediate blood-mediated inflammatory reaction (IBMIR) [22,23]. Clinical and animal studies have revealed that infiltration of leukocytes and an inflammatory response, together with activation of the complement and coagulation cascades leads to up to two thirds loss of islets within the first few days post transplantation [23]. Islet encapsulation is usually considered mainly as a technology to protect the islets from immune rejection whilst allowing glucose, oxygen and nutrient entry into and insulin release from the capsule-contained islets [24]. However, a biocompatible encapsulation technology that is focused as well, or instead, on restricting or preventing IBMIR and early islet destruction by host innate immune attack may be a major contribution to improving the clinical efficacy of islet transplantation.

Pancreatic islet encapsulation has traditionally included the use of microcapsules of, for example, alginate, which are produced via various droplet-generating processes [24]. Problems of conventional microencapsulation include hypoxic death of the cells and subsequent graft failure due to poor diffusion of oxygen and nutrients into the central cell mass, as well as incomplete immune protection and inadequate biocompatibility of the encapsulating materials, leading to non-specific protein adsorption and fibroblast overgrowth. In addition, microcapsules have large and therefore unsuitable transplant volumes for the intraportal transplantation route.

An attractive alternative strategy is to nano-engineer around individual islets a conformal nanocoating (nanoencapsulation) via deposition of nanofilms [25]. Conformal coating of isolated cells or cell clusters offers a mechanism for generating a selectively permeable and isolating layer close to the cell surface. The coating is very thin (nanometers), greatly reducing diffusional distances for O_2 and nutrients, thus potentially improving cell survival and function. More importantly for islet encapsulation and delivery is the fact that conformal coating does not substantially increase the encapsulated islet volume and is therefore compatible with intraportal transplantation. Several methods of conformal coating have been developed recently, including surface 'PEGylation' (attachment of poly[ethylene glycol]) and multilayer encapsulation, but some of these approaches suffer from poor biocompatibility of the synthetic encapsulating material, or incomplete surface coverage, neither of which is acceptable for islet encapsulation for clinical use.

LBL deposition, as noted above, generates a multilayered, thin film from oppositely charged species, deposited in succession on a solid support, which in this context is a cell or group of cells. A particular benefit of LBL technologies for cell encapsulation is that the layered structures can be fabricated with nanoscale precision (i.e. reproducibility), tuneable permeability and modifiable surface characteristics. Early studies with LBL encapsulation of islets employed cations such as poly(allylamine hydrocholoride) and anions such as poly(sytyrene sulphonate) [26], but encapsulation with passive barrier materials alone is generally insufficient to protect donor tissue from early loss or eventual rejection. However, multilayer films offer a versatile and facile platform for incorporating diverse biologically active agents into conformal coatings. Functional biomolecules of suitable charge like anticoagulants, complement regulatory proteins or anti-inflammatory agents can be incorporated into the linear polyelectrolyte multilayers during assembly, making the surface properties bioactive. Several studies have demonstrated that localised delivery of therapeutics to the site of grafts by conformal coatings may block coagulation, complement cascade and other islet destruction pathways, thereby significantly improving graft survival and reducing the islet mass necessary for reversing hyperglycaemia.

4.2. *Modification of coating materials*

4.2.1. *Phosphorylcholine-modified polysaccharide coatings*

We have developed an islet-coating protocol using alternate layers of phosphorylcholine (PC)-derived polysaccharides (chitosan or chondroitin-4-sulphate) and alginate as coating materials [27,28] (Fig. 2). We introduced the protein-repelling zwitterionic PC modification in the coating constructs to minimise interactions between islets and the environment. The PC moiety, which is a component of plasma cell membranes, confers hydrophilicity, haemocompatibility and resistance to non-specific protein absorption, thus potentially inhibiting the development of fibrosis, supporting endothelial cell growth and also carrying anticoagulatory properties, all of which are likely to enhance islet survival in vivo and encourage host integration.

Whereas naked, unencapsulated islets were rejected after 7–10 days when transplanted below the kidney capsule in an allogeneic diabetic mouse model, nanoencapsulated islets survived for at least 28–37 days in most animals, at which time nephrectomy was performed to examine islet histology. Moreover, nanoencapsulated islets maintained glycaemic control in the diabetic animals after an intraperitoneal glucose tolerance test [28].

4.2.2. *PEGylation*

PEGylation increases the biocompatibility of biomaterials by increasing hydrophilicity and preventing direct adsorption of proteins, as well as concealing surface structures, thereby preventing activation of the complement and coagulation cascade systems.

Teramura and Iwata [29] have developed a method for surface modification of pancreatic islets with PEG-lipid for improvement of graft survival after intraportal transplantation. Cell damage of PEG-islets after transplantation was suppressed, and the graft survival was significantly prolonged, compared with bare islets transplanted into livers of diabetic mice.

Conformal, nanothin PEG coatings have also been reported by the group of Chaikof et al. [30–32] where islets were encapsulated using LBL deposition of poly(L-lysine)-g-PEG-(biotin) and streptavidin (with poly [L-lysine]/alginate seed coating), and poly(L-lysine)-g-PEG copolymers and alginate (via polyion complex formation). Grafting of PEG chains to poly(L-lysine) was found to reduce the cytotoxicity of the polycations.

4.3. Modification of coating to incorporate therapeutic molecules

4.3.1. *Anti-Fas antibodies for preventing autoreactivation of T cells*

Surface-conjugation of apoptosis-inducing anti-Fas monoclonal antibodies to the surfaces of PEG-modified hydrogels embedding islets has been employed to provide a surface that actively attempts to locally down-regulate the autoimmune response by destroying autoreactive T cells against the grafted pancreatic islet cells [33].

4.3.2. *Thrombomodulin for regulating coagulation and inflammation*

Exposure of islets to fresh blood activates thrombotic reactions which are involved in the destruction of transplanted islets. Chemoselective conjugation of antithrombotic azido-functionalized thrombomodulin to pancreatic islets was achieved by ligation to a surface-bound bifunctional PEG linker [34].

Thrombomodulin was also immobilized on islet surfaces through streptavidin–biotin interactions. The presence of the tethered thrombomodulin resulted in a significant increase in the production of activated protein C, with a reduction in islet-mediated thrombogenicity. The thrombomodulin-reengineered surface reduced donor cell-mediated procoagulant and proinflammatory responses [35].

4.3.3. *Heparin coating for inhibiting IBMIR*

All currently identified systemic inhibitors of the IBMIR are associated with a significantly increased risk of bleeding or other side effects. To avoid systemic treatment, a continuous heparin coating to the islet surface has been investigated as way of rendering the islet graft more blood biocompatible. A biotin/avidin technique was used to conjugate preformed heparin complexes to the surface of pancreatic islets [36]. This endothelial-like coating was achieved by conjugating 40 IU heparin per clinical islet transplant. Both in an in vitro model and in an allogeneic porcine model of clinical islet transplantation, this heparin coating provided protection against IBMIR. Heparinized islets cultured for 24 h had unaffected insulin release after glucose challenge, and heparin-coated islets normalised glycaemia in diabetic mice in a manner similar to untreated islets.

4.3.4. *Urokinase for inhibiting IBMIR*

PEG-lipid and PEG-urokinase have been immobilized onto islets through hydrophobic interaction and streptavidin/biotin interaction or DNA hybridization [37–42]. Efficacy of surface modification for the inhibition of IBMIR was proven using a syngenic mouse transplantation model. Outcomes evaluated were blood insulin levels immediately after transplantation, blood glucose levels, and histochemical analyses of transplanted islets within the liver.

4.3.5. *Heparin/sCR1 for inhibiting IBMIR*

Human soluble complement receptor 1 (sCR1) and heparin have been co-immobilized onto the surfaces of islet cells in a LBL manner [42,43]. sCR1 molecules carrying thiol groups were immobilized through maleimide-PEG-phospholipids anchored in the lipid bilayers of islet cells. Heparin was

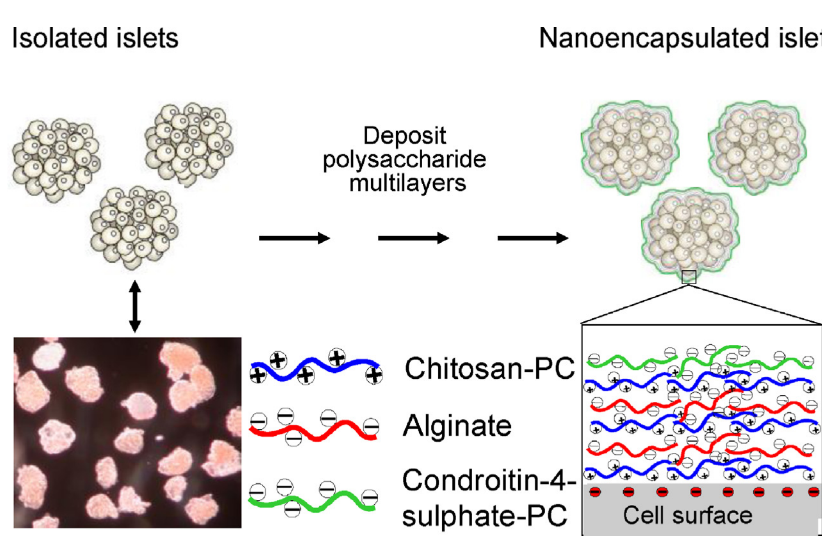

Fig. 2 – Nanoencapsulation of pancreatic islets by layer-by-layer deposition of ultra-thin polyelectrolyte nanofilms. Islets were coated with a cocktail of chitosan-phosphorylcholine (PC), alginate and chondroitin-4-sulphate-PC. Adapted from ref. [28].

immobilized on the sCR1 layer via the affinity between sCR1 and heparin, and additional layers of sCR1 and heparin were formed in a similar way layer-by-layer.

4.3.6. *Extracellular matix peptide*

Extracellular matrix IKVAV peptide has been immobilized on the surface of pancreatic islets through strain-promoted azide–alkyne cycloaddition with cell surface azides [44]. The cyclooctyne-derivatized IKVAV peptide conjugate enabled efficient modification of the pancreatic islet surface in less than 60 min. The ability to bind peptides at controlled surface densities was demonstrated in a quantitative manner using microarrays.

5. Nanoencapsulation of insulin for controlled release

5.1. *Oral insulin*

Various delivery strategies for overcoming the formidable barriers of enzymatic digestion and poor absorption of orally delivered insulin have been explored, including using carrier systems in which insulin is entrapped into particulate structures such as microspheres or nanospheres [45]. These insulin-loaded nano/microparticles can have limited colloidal stability and readily dissociate and dissolve in the acidic gastric conditions. Although the therapeutic effect is fast and intense after oral administration of, for example, insulin–chitosan nanoparticles, the oral bioavailability of the drug from an immediate release dosage form is poor and highly variable. Designing a dissolution barrier allowing control over the release of insulin from the microparticles by multilayer encapsulation could help overcome these problems.

5.1.1. *Chitosan/heparin multilayer-coated insulin particles*

We have been researching a novel application for LBL technology to nanoencapsulate insulin-incorporated micro-colloids with very high content of the bioactive protein (90% by wt) and controllable drug release rate (Song, Zhi, Pickup, unpublished observations). Insulin–chitosan micro-colloids were surface-coated by multilayered shells comprising chitosan and heparin. By adding PEG in the aqueous assembly solutions, the insulin loss can be dramatically reduced during deposition steps and stable micro-colloidal capsules are achievable. Deposition of chitosan–heparin ultrathin films onto the insulin particles via polyion complex formation was found to enhance the particle colloidal stability at acidic pH conditions and control loading/release characteristics, thereby achieving more efficient delivery of oral insulin (Fig. 3). Such chitosan–heparin multilayer encapsulated insulin–chitosan complexes significantly lowered blood glucose levels in diabetic mice, while the insulin capsules alone caused no reduction.

5.1.2. *Chitosan/albumin multilayers*

Chitosan and albumin layers have been used to stabilize insulin nanoparticles formed along with calcium alginate, dextran sulphate and a poloxamer (a triblock copolymer) [46]. Insulin nanoencapsulation reduced plasma glucose levels to 40% of the basal values with a sustained hypoglycaemic effect over 24 h. Pharmacodynamic and pharmacokinetic parameters were evaluated at a dose of 50 IU/kg nanoencapsulated insulin, and 13% oral bioavailability was obtained.

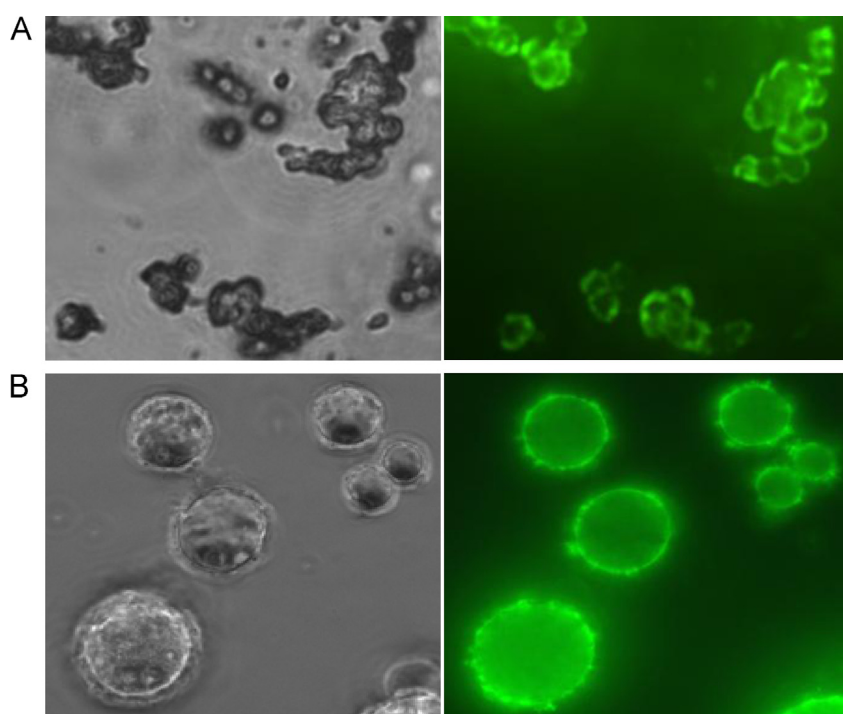

Fig. 3 – Light and fluorescent images of nanoencapsulated insulin–chitosan (A) and insulin (B) particles at pH 2.0. Left: brightfield; right: fluorescence. At pH 2.0, the insulin alone capsules are empty, caused by the insulin particle dissolution. For encapsulated insulin–chitosan particles, the particles remained intact.

5.1.3. *Fe^{3+}/dextran/protamine multilayers*

Insulin-loaded microcapsules have also been prepared via LBL complex formation of oppositely charged Fe^{3+} and dextran sulphate (DS) onto the surface of insulin microparticles [47]. Fe^{3+} was combined with DS via both electrostatic interaction and chemical complexation, leading to the formation of a stable complex of Fe^{3+}/DS. Protamine was used as the outermost layer of the microcapsules to facilitate nuclear delivery. The microcapsules successfully entrapped insulin with efficiency of 70% and drug loading content of 46%. The insulin-loaded microcapsules significantly improved glucose tolerance for up to 12 h (insulin-loaded microcapsules with 10 bilayers). Moreover, the microcapsules with protamine as the outermost layer displayed a prolonged and stable glucose-lowering profile in vivo over a period of over 6 h, compared with Fe^{3+} as the outermost layer.

6. Conclusions

A significant body of research now shows that multilayer nanoencapsulation has promise in developing improved glucose sensors and enhancing islet survival after transplant. This work now needs to be extended to clinical testing. Nanoencapsulated insulin preparations also show promise as a potential oral delivery system, but this research is at an earlier stage and needs more extensive testing in animals before progressing to human evaluation.

Conflict of interest

The authors declare that they have no conflict of interest.

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