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REVIEW ARTICLE

Nanomedicine and its potential in diabetes research and practice

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Summary

Nanomedicine involves measurement and therapy at the level of 1–100 nm. Although the science is still in its infancy, it has major potential applications in diabetes. These include solving needs such as non-invasive glucose monitoring using implanted nanosensors, with key techniques being fluorescence resonance energy transfer (FRET) and fluorescence lifetime sensing, as well as new nano-encapsulation technologies for sensors such as layer-by-layer (LBL) films. The latter might also achieve better insulin delivery in diabetes by both improved islet encapsulation and oral insulin formulations. An 'artificial nanopancreas' could be an alternative closed-loop insulin delivery system. Other applications of nanomedicine include targeted molecular imaging *in vivo* (e.g. tissue complications) using quantum dots (QDs) or gold nanoparticles, and single-molecule detection for the study of molecular diversity in diabetes pathology. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords nanomedicine; glucose sensors; fluorescence; nanoparticles; insulin; single-molecule

Introduction

Nanotechnology is the measurement and manipulation of material at the level of 1–100 nanometres (nm), 1 nm being 10^{-9} or one billionth of a metre (*nanos*, Greek, 'dwarf'). When this science is applied specifically to the problems of medicine, it is called 'nanomedicine' [1,2].

The nanomedicine scale conventionally excludes at the lower end atoms, which have a size of about 0.1 nm, and at the upper end biological entities such as bacteria (1000–10 000 nm) and body cells (e.g. 10 000 nm for a white blood cell). Clearly, the body has configured many of its biocomponents as nanostructures, including proteins, mitochondria, ion channels, membranes, secretory granules, lysosomes and so on, but many new nanomaterials and structures are now being manufactured that might be of use in medicine, including nanoparticles, capsules, films and tubes, and complex molecules such as fullerenes (a new allotrope of carbon containing, in its original form, 60 carbon atoms arranged symmetrically as a molecular ball of diameter about 1 nm [3]).

Nanomedicine can be classified into (1) *measurement (*or *'nanometrology'*), which concerns either measuring very small amounts of analytes (e.g. single molecules) or using very small-sized devices for measuring (e.g. sensors within a cell), or (2) *therapy*, as all of the manipulations and constructions of materials at the nano-level ultimately concern therapies (e.g. membranes and coatings for more biocompatible implants or vehicles for drug delivery), if they do not concern measurement (e.g. constructing nanoscale devices for monitoring analytes in or out of the body).

Why are we interested in nanomedicine?

Some of the potential advantages of nanoscale research and its clinical applications are fairly obvious, such as small size allowing unprecedented access to target areas within the body (e.g. nanostructures and devices for imaging, analysis, treatment or repair inside diseased tissues and cells), and the assay of very small amounts of bioanalyte might allow earlier, more sensitive diagnosis. But much of the interest in nanotechnology is for the less obvious reason that the nature of some materials is altered in unexpected ways as size is reduced, called 'quantum effects', producing changes in properties such as electrical conductivity, strength, colour and reactivity. For example, carbon, which is soft and malleable as graphite becomes, in the form of carbon nanotubes (~1.5 nm in diameter), flexible, resilient and stronger than steel, as well as fluorescent and conducting electricity with virtually no resistance [4,5].

The clinical need and the vision for nanomedicine in diabetes

Applications of nanotechnology in diabetes are in their infancy. As is often the case in the development of medical technology, advances in fields such as biomaterials, analytical science and engineering are occurring faster than, and often without much reference to, their translation into routine clinical practice. It is useful therefore to review some of the outstanding problems in diabetes care and the potential nanomedicine has for their solution (Table 1).

Glucose monitoring

It is widely accepted that there are major problems with conventional finger-prick capillary blood glucose self monitoring [6]: it is painful (leading to non-compliance), it cannot be performed when the patient is sleeping or driving a motor vehicle (times when the patient is especially vulnerable to hypoglycaemia) and, because it is intermittent, it can miss dangerous fluctuations in blood glucose concentrations between tests. The ideal blood glucose monitoring would therefore be continuous and non-invasive. Several subcutaneously implanted needle-type enzyme electrodes or microdialysis probes for continuous glucose monitoring are now marketed or close to market [7–9]. But such devices are still limited by a relatively short duration of use (up to about 7 days currently) and impaired responses and unpredictable signal drift *in vivo*, which necessitates calibration against capillary glucose tests and contributes to sensor inaccuracies. The repeated insertion of the sensor probe is also semi-invasive. How can nanomedicine help solve these problems?

One vision that might meet the need for improved *in vivo* glucose monitoring is a 'smart tattoo' composed of glucose-responsive, fluorescence-based nanosensors implanted into the skin but interrogated from outside the body, thus making monitoring non-invasive (Figure 1). Sensors that use fluorescence for detecting analyte changes have some advantages compared to the more usual implanted electrochemical electrodes, as they should not be susceptible to electroactive tissue interferents that contribute to the instability of present sensors, and because near infrared (NIR) light with a wavelength above about 600 nm passes through several centimetres of tissue, allowing implantation and non-invasive measurement at the body surface.

A number of biological or artificial receptors for glucose have been described, which can transduce glucose concentrations into changes in fluorescence, including lectins, enzymes, bacterial binding proteins and boronic acid derivatives [10], and which might be engineered as nanosensors. With the plant lectin concanavalin A (Con A), which has four binding sites for glucose, sensing can be based on the competitive binding to Con A of either glucose or a labelled carbohydrate derivative such as dextran [11] or Sephadex beads [12].

Table 1. Some problems in diabetes and possible nanomedicine solutions

Measurement problems	Nanometrology solutions
Continuous blood glucose monitoring:	
Stable implanted enzyme electrodes	Biocompatible nanofilms
Non-invasive monitoring	'Smart tattoo' of glucose nanosensors
Improved diagnosis/monitoring complications:	
Targeted molecular imaging	NIR QDs, gold nanoparticles
Understanding mechanisms	Single-molecule detection
Therapy problems	**Nanotherapeutic solutions**
Improved insulin delivery:	
Islet cell transplantation	Islet nanoencapsulation
Oral insulin	Insulin nanoparticles
Closed-loop insulin delivery	'Artificial nanopancreas'

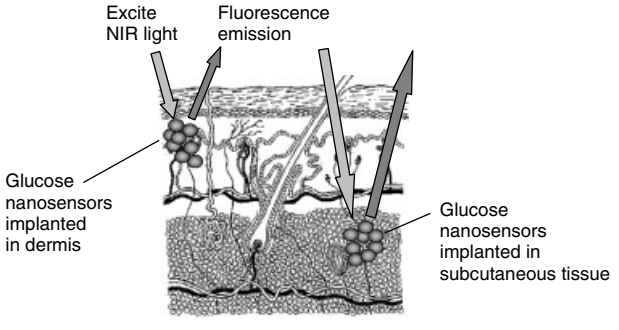

Figure 1. The 'smart tattoo' concept for non-invasive glucose sensing in diabetes. Nanosensors consisting of encapsulated, fluorescently labelled glucose-receptor molecules (e.g. glucose-binding protein [GBP]) are implanted in the skin (dermis or subcutaneous tissue) and can be excited using NIR light and the fluorescence detected from the skin surface

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For example, we described a glucose assay in which Con A was covalently labelled with the highly NIR-fluorescent protein allophycocyanin (donor), and dextran was labelled with the non-fluorescent dye, malachite green (acceptor) [11]. Addition of glucose displaces dextran from Con A, thereby reducing fluorescence resonance energy transfer (FRET) between the donor and acceptor and the measured fluorescence lifetime.

Some enzymes might act as nanosensors: hexokinase shows a 25% reduction in its intrinsic fluorescence on addition of glucose [13]. This is attributable to quenching of one of the four tryptophan residues in the enzyme, which occurs as glucose binding induces a conformational change in the protein. Hexokinase entrapped in the nanopores of silica sol-gel can monitor glucose in serum as the interferents in blood which quench the fluorescence of hexokinase in solution are excluded, and the increased K_d of the encapsulated enzyme (from 0.3 mM in solution to 12.5 mM in gel) is suitable for clinical glucose monitoring [13]. However, excitation/emission of hexokinase tryptophan residues is achieved at 295/330 nm and would prevent deep skin impregnation of such a sensor.

An alternative conformation-sensitive receptor is bacterial glucose/galactose-binding protein (GBP), which has been the subject of a number of studies on fluorescence sensing of glucose [14–17]. GBP is formed of a single polypeptide chain that folds into two domains connected by a hinge, and glucose binding is accompanied by a large conformational change and closing of the domains around the glucose, similar to hexokinase. This gives opportunities for fluorescence sensing of glucose in two ways (Figure 2). Firstly, changes in FRET can be monitored between a site-specifically attached fluorophore donor and an acceptor conjugated to different parts of the GBP molecule. For example, with green fluorescent protein fused to the C terminus and yellow fluorescent protein fused to the N terminus of GBP, glucose binding and altered tertiary structure causes separation of the fluorophores and reduced FRET (increased fluorescence intensity), proportional to glucose concentration [17]. We also demonstrated reduced FRET on addition of glucose to GBP fluorescently labelled with Alexa Fluor dye linked at the N terminus and an acceptor (QSY 7) near the binding site [18].

A second sensing strategy with GBP is to monitor glucose-dependent changes in fluorescence of an environmentally sensitive dye (i.e. one where fluorescence is low in a polar environment and high in a non-polar one) linked to a suitable site in the protein. For example, we found that with the environmentally sensitive dye, badan (ex 400 nm, em 550 nm), covalently attached to a cysteine residue introduced by site-directed mutagenesis at position 152 of GBP, glucose addition caused a 300% increase in fluorescence, likely due to folding of the protein around the dye producing a less polar environment [18].

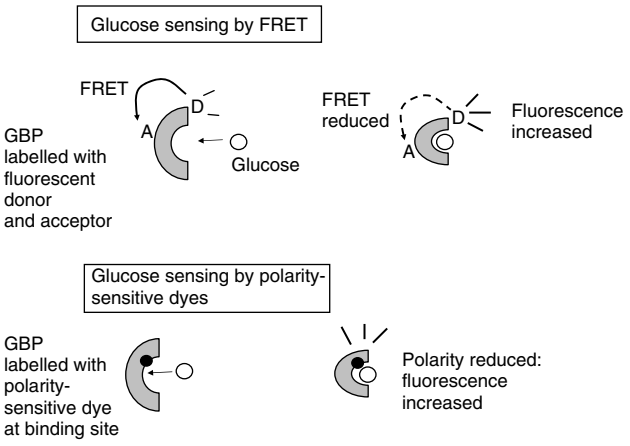

Figure 2. Two strategies for sensing glucose using GBP. Top: changes in FRET occur as glucose binding induces a conformational change in GBP, with fluorescent donor and acceptor moving further apart. Bottom: an environmentally sensitive fluorophore is attached to an amino acid residue close to the binding site, and glucose binding closes the polypeptide chain around the label, causing local polarity to decrease and fluorescence to increase

The next challenges for clinically usable fluorescence-based glucose nanosensors include extending the excitation and emission of GBP-based sensors into the NIR range and here, Thomas *et al*. have made significant progress by synthesizing a cysteine-reactive derivative of the environmentally sensitive NIR dyes, Nile Red [19], and benzothiazolium squarine [20], that have been conjugated to GBP.

Further progress must be made in encapsulation of glucose sensors in a form that can be implanted in the body and yet maintain functionality – avoiding degradation, denaturation, leakage and foreign body reactions, while retaining glucose access and detectable signal change. An example of a technology that may be appropriate for this is electrostatic layer-by-layer (LBL) nanoassembly of capsules composed of multi-layers of polymer films [21–23]. These are proving to be both stable and versatile, and with tunable permeability.

In the case of glucose sensors, LBL encapsulation has been achieved by two approaches. Trau and Renneberg [22] described sequential adsorption of oppositely charged polyelectrolytes (polyallylamine hydrochloride and polysodium styrene sulfonate) onto microcrystals of glucose oxidase. Zhi and Haynie [23] reported a template-supported LBL method for capsule construction in which glucose oxidase was first adsorbed onto colloidal particles of calcium carbonate, followed by stepwise additions of charged polypeptides (polylysine and polyglutamic acid), and finally dissolution of the template in ethylenediaminetetraacetic acid (EDTA) (Figure 3). Addition of 50% polyethylene glycol (PEG) to the layer solutions increased glucose oxidase retention on the template during the LBL assembly. The typical layer thickness for each bilayer was 1.5 nm.

LBL deposition of nanofilms may also offer opportunities for improving the biocompatibility and operating

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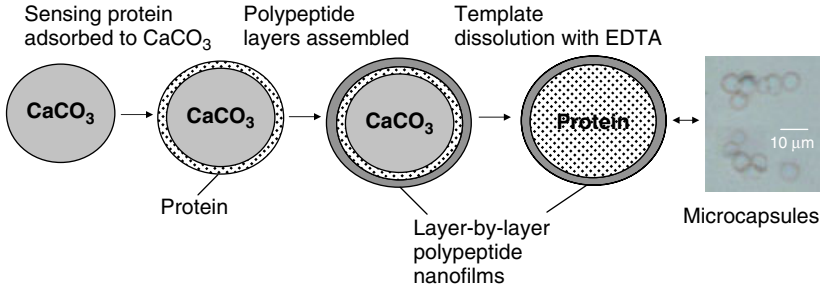

Figure 3. Encapsulation of a glucose-sensing protein in nanoengineered microcapsules. The protein is adsorbed onto a template of calcium carbonate, alternating layers of poly-L-lysine and then poly-L-glutamic acid are applied, followed by dissolution of the template using EDTA

stability of the existing needle-type implantable amperometric glucose sensors, e.g. by incorporation of anti-inflammatory agents, extra-cellular matrix mimics and other factors in the layers.

Improved insulin delivery

Islet cell implantation

The main barriers to the successful treatment of type 1 diabetes by islet cell transplantation are the insufficient availability of islets and the poor survival of implanted islet cells due to hypoxia and immune rejection. To overcome these problems, islet encapsulation attempts to create a 'bioartificial pancreas', isolating the islets (which might include animal islets or insulin-producing cell lines) in a permselective membrane that allows glucose-dependent insulin release from the islets and nutrient access across the membrane, while excluding the large proteins and cells of the immune system [24,25]. Several encapsulating materials have been used, most often alginate/polylysine, and also agarose, polysulfone, methacrylates, PEG, polyvinyl alcohol and other materials.

In spite of more than 40 years of research, islet encapsulation has yet to reach clinical practice. Limitations include hypoxic death of the cells due to poor diffusion of oxygen and nutrients into the central cell mass, poor biocompatibility of the membrane leading to fibrotic overgrowth and insufficient immunoprotection, particularly due to incomplete membrane covering [24,25]. This last problem can lead to the release of islet cytokines and the chemotaxis of macrophages around the capsule, with 'walling off' and cell death due to macrophage-produced nitric oxide diffusing through into the capsule.

LBL encapsulation with multi-layers of alternating positive- and negative-charged polymers has been described earlier in the context of nanosensors [23], and has also been recently applied to islet cells [26,27]. The potential advantages are complete coverage, the nanothickness of the membrane is associated with an enhanced response time and better nutrient access and tunability of permeability are possible by controlled layer thickness and composition. Enhanced biocompatibility and survival might be achieved by incorporating mimics of the extra-cellular matrix onto the capsule or by local immunosuppressant release. Krol *et al*. [26] reported polyelectrolyte LBL islet encapsulation, including polyallylamine hydrochloride/polystyrenesulfonate, and Teramura *et al*. [27] used LBL membranes of polyvinyl alcohol conjugated to a single layer of PEG-phospholipid at the islet surface.

In provisional studies, we have demonstrated LBL encapsulation of islet cells and β-cell lines (MIN 6 cells) using nanoengineered polylysine/polyglutamic acid membranes, which maintain glucose-responsive insulin secretion (Figure 4).

Oral insulin

Insulin cannot be given unaltered by mouth because it is denatured by the adverse pH and undergoes proteolysis by enzymes in the gut, and because the gut has low permeability for large molecules [28,29]. (The gut motility is also impaired in diabetes, which adds delayed absorption and unpredictability to the problems). Some of the technologies of nanomedicine might offer ways of protecting insulin and enhancing its absorption.

There is a long history of attempts to encapsulate insulin as an oral formulation, including the study of liposomes starting in the 1970s [30]. These lipid bilayer-bounded

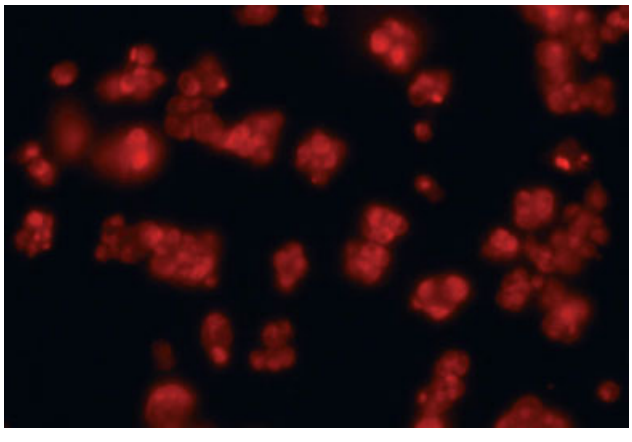

Figure 4. Fluorescence microscopy image of encapsulated beta cells. Three layers of poly-L-lysine and poly-L-glutamic acid were coated on clustered MIN-6 cells. The outmost layer was Alexa Fluor 647-labelled poly-L-lysine, used to demonstrate the polypeptide encapsulation

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vesicles were found to inhibit proteolysis, undergo endocytosis into the endothelial cells and lower blood glucose levels, but the response was not predictable or dose-dependent. Among the early nanoparticle studies, Damgé *et al*. [31] found that biodegradable cyanoacrylate particles loaded with insulin caused dose-dependent and prolonged glucose lowering in rats. Recent nanoparticle insulin formulations, which have been shown to produce glucose lowering in animals include clinically well-accepted polycaprolactone/polyacrylic polymers [32] and insulin incorporated with the polycationic polysaccharide, chitosan, which is mucoadhesive (prolonging residence time in the gut) and enhances permeability by disrupting tight junctions between gut epithelial cells [33]. LBL encapsulation (see glucose nanosensors and islet encapsulation given earlier) of insulin should be explored as it offers the possibility of protection and incorporation of absorption enhancers in the layers.

Closed-loop insulin delivery: an 'artificial nanopancreas'?
The classical concept of an artificial endocrine pancreas is a bedside or wearable electromechanical device consisting of a glucose sensor implanted in the body (or detecting glucose in blood or tissue fluid withdrawn from the body), and coupled to an insulin infusion pump via a computer. Using algorithms to relate measured glucose to the insulin infusion rates that are needed to maintain normoglycaemia, it therefore provides closed-loop control of insulin delivery [34]. This type of device has been under development since the 1960s and although significant progress is being made, many consider that the present sub-optimal performance of implanted glucose sensors (see earlier) means that unsupervised and safe routine operation at home is a significant challenge. An alternative approach might be a non-mechanical, closed-loop system in which glucose sensing is coupled to insulin delivery by molecular or nanostructured components: an 'artificial nanopancreas'.

Early attempts at molecular-level, glucose-regulated insulin delivery included glucose displacement of glycosylated insulin from Con A [35], and incorporation of insulin in a pH-sensitive membrane containing immobilized glucose oxidase [36]. In the latter, glucose was oxidized to hydrogen peroxide and gluconic acid, generating H^+ ions, opening membrane pores and releasing insulin. Such systems were thought to be limited by the slow release kinetics of insulin (though this might not be a problem if used only in the basal mode and not at meal times) and, in the case of the pH-sensitive membranes, buffering of the pH changes by the high buffer capacity of plasma and interstitial fluid.

We now have several of the nanocomponent parts of an artificial β-cell, including well-understood glucose receptors such as GBP which do not involve pH change, nanoencapsulation methodologies for glucose sensors and insulin, tools for constructing and controlling membrane permeability and its biocompatibility. The grand challenge for the coming decades is to interface these functionalized nanocomponents to form an integrated β-cell mimic.

Improved diagnosis and prediction of diabetes and its complications

Targeted molecular imaging
The need for *in vivo* imaging of the location, character and quantity of dysfunctional tissue over a period of time is well established in cancer medicine for detecting and monitoring primary and secondary tumours, but has not yet had much impact in diabetes. Among the possible opportunities are better diagnosis and monitoring of diabetes complications like retinopathy and atherosclerosis, and pathological processes such as islet inflammation in type 2 diabetes, by the sensitive imaging of dysfunctional tissues.

Targeted imaging is achieved by labelling molecules such as antibodies that have an affinity for the structures of interest, say with a fluorescent probe that can be imaged within the body. In this context, there has been much interest in quantum dots (QDs) as *in vivo* molecular probes [37]. QDs are nanosized (2–10 nm) colloidal crystals of semi-conductors such as cadmium selenide, coated with a shell to improve optical properties (often zinc sulfide), and a cap such as silica to improve solubility. Because of quantum confinement effects, QDs have uniquely useful properties as probes, namely stable and very bright (high quantum yield) fluorescence that is not subject to photobleaching, and a broad excitation and narrow waveband of fluorescence emission that depends on the size of a particle. For example, a large CdSe QD (7.5 nm) can be excited at any wavelength from UV to the upper end of the visible spectrum and emits fluorescence with a narrow range around 650 nm in the NIR region. NIR QDs have been used for *in vivo* imaging of lymph nodes, tumours and blood vessels in animals [37,38], but targeted imaging in humans of, say, diabetes-related lesions will need smaller dots that can pass from the blood to the tissues and that can be excreted from the body, with avoidance of non-specific take-up into the reticuloendothelial system, and more knowledge and assurances about the toxicity profile.

In contrast to QDs, noble metal nanoparticles are biocompatible, the surface plasmon resonance colour of gold nanoparticles being used for some time to visualize antigens in optical microscopy [39]. Although the photoluminescence of gold nanoparticles is too weak for imaging using one-photon excitation (OPE), recent work has shown that surface plasmons in asymmetric gold nanoparticles (nanorods) generate more intense photoluminescence under two-photon excitation (TPE) [40] than aromatic fluorophores. TPE is a non-linear absorption process generated using ultrafast (typically $\leq$100 femtosecond, 1 fs $= 10^{-15}$ s) laser pulses (usually a Ti : sapphire laser at $\sim$800 nm) and is increasingly used in fluorescence lifetime spectroscopy [41,42] as well as fluorescence microscopy [43], bringing to the

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latter advantages over OPE of reduced photobleaching, greater penetration depth and higher spatial resolution. Although TPE applied to nanomedicine is still embryonic, it has recently been used to image cancer cells in tissue phantoms down to 75 μm depth [44].

Single-molecule detection (SMD)

Detection of single molecules is the ultimate in the sensitive assay of analytes but it also has significant potential in medicine for another reason; because, unlike conventional clinical chemistry assays that measure the average concentration of molecular ensembles, it allows assessment of the pattern distribution of single molecular species that may differ from each in form or function and between health and disease [45]. No studies of single-molecule detection (SMD) as applied to the problems of diabetes have yet been reported, but the first use of SMD in diabetes is likely to be in research rather than clinical practice, for example, for investigating the molecular profile of biomarkers of complications as an aid to understanding pathology, though this may eventually lead to new ways of predicting and monitoring disease.

The technologies for SMD have been reviewed recently [46], and include manipulation-related techniques such as atomic force microscopy, surface-enhanced Raman spectroscopy [47] and various forms of fluorescence microscopy such as confocal, total internal reflection and scanning near field optical microscopy (SNOM). The advantages of fluorescence for this application, including single-photon sensitivity, non-invasive and non-destructive detection and the several aspects of fluorescence, which can be dependent on molecular structure, are excitation and emission wavelength, intensity, lifetime and polarization.

Much of the toolbox needed to bring single-molecule science [48] into nanomedicine is still being developed, particularly with respect to the relevant nanotechnology, nanometrology and biocompatibility issues. For example, the use of hydrated sol-gel nanopores [13] to entrap and protect metabolite-specific proteins for detecting non-fluorescent metabolites (such as glucose sensed by hexokinase) can in principle be developed to operate at both the single-molecule limit and in lab-on-a-chip sensors, the latter facilitated by the new generation of semi-conductor optical sources now available for exciting protein intrinsic fluorescence [49] as well as labels.

Potential toxicity of nanomaterials

There is as yet no reason to think that nanomaterials pose a certain toxic threat or that any possible harmful effects cannot be assessed and managed as is done for any new pharmaceuticals, diagnostics or medical materials. However, as there is a lack of information and research on nanomaterial toxicity, it is important to consider the potential health and safety issues. The determinants of particle toxicity are known to be the large surface area and chemical reactivity in relation to small size (and thus the ability to generate reactive oxygen species) and the ability to penetrate tissues and cells [50]. Thus, nanoparticles are likely to be more hazardous than the same chemicals in larger form, and free particles more toxic than fixed ones [51].

The potential cytotoxicity of QDs is an example to consider, because at high concentrations harmful effects on embryo development and cell viability and function have been recorded [37]. Nanoparticles such as QDs most likely need to be passivated with less toxic materials to improve biocompatibility, for example with silica [51]. The safety profile of other nanomaterials discussed in the review such as polypeptide capsules has yet to be fully investigated.

Among the issues that need to be resolved are whether existing regulatory rules are sufficient to cover new and future nanomaterials and to protect both humans and the environment. Lack of information creates uncertainty and anxiety about the possible harmful effects of nanotechnology and there is a major need for continued research into any potential health hazards [52].

Conclusions

Nanomedicine is at a very early stage, but progress is rapid, translational, expansive and multi-purpose. Diabetes has many remaining problems; nanomedicine is likely to be a key technology for solving many of them.

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Conflict of interest

None declared.

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