ABSTRACT

The present invention relates to a method of preparing fluorescent polysaccharide based pH responsive compositions.

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We claim:

1. A method of preparing fluorescent polysaccharide based pH responsive compositions comprises:
   a) atomization
   b) encapsulation
2. According to the method of claim 1, the atomization method utilizes an encapsulation unit
3. According to the method of claim 1, the FITC-dextran fluorescent dye is encapsulated within calcium alginate microspheres.
4. A method of preparing pH responsive composition for detection of urea comprising of FITC-dextran and Urease co-immobilized polysaccharide (Ca-alginate) microspheres.
5. According to the method of claim 4, the composition of pH responsive matrices consist of (FITC-dextran) fluorophore encapsulated calcium alginate microspheres.
6. According to the method of any of claim 1 and 4, the pH responsive fluorescence changes were detected by exposing solutions of different buffer solutions and measuring the changes using fluorescence spectroscopy.
7. According to the method of any of claim 1 and 4, different combinations of FITC-dextran and urease loading were optimized to obtain desirable sensitivity and range of biosensing.
8. According to the method of any of claim 1 and 4, water soluble polysaccharides but not limiting to like alginate, gelatin, chitosan are used for crosslinking.
9. According to the method of any of claim 1 and 4, carriers for biomolecules are selected from but not limited to reagents like calcium salts, glutaraldehyde, or tripolyphosphates.
10. According to the method of any of claim 1 and 4, gelling agents for cross linking of alginates are selected from but not limited to metal cations such as metal cations of barium, lead, copper, strontium, cadmium, calcium, zinc, nickel and aluminium or a mixture of any of the above.
11. According to the method of any of claim 1 and 4, counter ions are selected from but not limited to carbonates, sulphate, chlorides, acetates
12. According to the method of any of claim 1 and 4, the composition developed is capable of detecting pH changes in the range (0-10 units) and urea changes from (0-50 mM) which cover the physiological and pathological levels of pH and urea concentrations.

13. According to the method of claim 12, wherein the composition can be transformed into an important sensing assay for use as a urea biosensor and used in the development of point of care devices for diagnosis of diseases and health monitoring of disease.
14. According to the method of claim 13, the sensor is based upon measurement of the pH change produced in an aqueous environment by the products of the enzyme-catalyzed hydrolysis of urea.
15. According to the method of any of claim 1 and 4, production of microspheres includes: blending of water soluble polysaccharide into water which forms the basis of polymeric material; transferring a solution of alginate and active molecules like fluorescent dyes and enzymes to a syringe in the syringe pump.
16. According to the method of claim 15, wherein the said solution is sprayed using an encapsulation unit through a spray nozzle based on air flow system.
17. According to the method of claim 16, wherein the flow rate, height of the nozzle head and air pressure are varied and optimized to obtain different sizes of microspheres
18. According to the method of claim 17, wherein gelation occurs through this process leads to hardened microspheres.

Dated at Mumbai this May 10, 2013

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COMPLETE SPECIFICATION

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TITLE OF THE INVENTION

FLUORESCENT POLYSACCHARIDE BASED PH RESPONSIVE COMPOSITIONS AND METHODS OF PREPARATION THEREOF

10 ## FIELD OF THE INVENTION

The present invention relates to a method of preparing fluorescent polysaccharide based pH responsive compositions.

15 ## BACKGROUND OF THE INVENTION

Estimation of pH and urea is essential in the field of medicine to study proper functioning of important organs like kidney. Kidney is the site of hormone production and secretion, acid-base homeostasis, fluid and electrolyte regulation and waste product elimination. Uremia more commonly develops with chronic renal failure (CRF) or the later stages of chronic kidney disease
20 (CKD) but it also may occur with acute renal failure (ARF) if loss of renal function is rapid. A high protein diet, drug use, increase in protein breakdown due to an infection, surgery, cancer or trauma for example, can cause uremia. Each of these potential causes of uremia makes the liver produce excessive amounts of urea, which may appear in the blood stream.

25 In routine procedures urea determination in biological fluids is carried out with chemical reagents and enzymatic methods. Uremia is a clinical syndrome associated with fluid, electrolyte, and hormone imbalances and metabolic abnormalities, which develop in parallel with deterioration of renal function. The term uremia or urea in the blood is the clinical condition associated with renal function. Uremia is a potentially fatal condition that demands immediate
30 treatment. Treatment options for uremia include kidney transplant, dialysis and other treatments typically associated with kidney failure. In some cases, uremia may be alleviated by making

specific dietary changes or by otherwise eliminating the underlying cause of the disorder. An increase in the general population with a larger proportion of elderly persons and a greater percentage of hospitalized patients receiving treatment in the Intensive Care Units (ICU) have driven a phenomenal increase in ICU costs, which may be 20% of all hospital costs. Surgery was found to be the main reason for ICU admittance; acute/chronic cardiovascular disorders led to treatment in the ICU in 78% of the patients, followed by disorders of the nervous system (29%), gastrointestinal system (25%), and severe infections (28%). The average period of stay was 6.2 days as described in *Acta Anaesthesiol Scand Suppl*, 84 (1987) 3-19.

Urea is an important marker for diseases related to kidney and liver conditions. With the advent of developments in biosensors, a urea biosensor appears more feasible to help patients monitor the urea levels using a point of care device. The FITC-dextran loaded microspheres act as a pH sensor and when urease is co-immobilized with FITC-dextran in the alginate microspheres it will act as a urea biosensor. Both pH and urea biosensors can be beneficial markers for detection and monitoring of kidney diseases.

Various methods are reported in the literature for the preparation of compositions comprising chemically sensitive particles for measuring analytes and methods thereof. Methods involving encapsulation is apparently beneficial because of their simple operation and high efficiency. Microsphere and biomolecule based materials are used as novel materials for such compositions. Various references also describes on compositions and methods for analyte detection including US Patent No. 6,485,703, US Patent Application Publication No. 2011/0262363, PCT Publication No. WO 2010/142960, US Patent Application Publication No. 2012/0231443, US Patent Application Publication No. 2003/0230819, EP Patent No. 1975230.

Further fabrication and evaluation of fluorescent based alginate microsphere for analyte detection are described in XVII International Conference on Bioencapsulation, 2009, IIT Bombay, *International Journal of Pharmaceutics*, 2005, 304(1-2), 18-28, *Analyst*, 2010, 135(10), 2620-2628, Thesis, Department Of Biosciences and Bioengineering, 2011, Conference Proceedings, IEEE Engineeering In Medicine & Biology Society, 2009, 4098-4101, *Biotechnology and Bioengineering*, IIT Bombay, 2009, 104(6), 1075–1085. Furthermore few

references describing the recent development in optical fiber biosensors including *Sensors*, 2007, 7(6), 797-859, *Nano/Molecular Medicine and Engineering* (NANOMED), 2009, 129-132, *Sensors and Actuators B: Chemical*, 2012, 173, 882–889 are reported earlier.

Subsequently, there is a growing demand for greater accuracy, efficiency and cost-effectiveness in detection and treatment methods used in critical care units such as in dialysis monitoring. Hence, detectors for measurement of the pH change, using an appropriate pH sensitive dye, produced in the aqueous environment by the products of the urease catalyzed hydrolysis of urea has to be developed, wherein the FITC-dextran loaded microspheres act as a pH sensor and when urease is co-immobilized with FITC-dextran in the alginate microspheres will act as a urea biosensor in clinical applications to detect analytes.

SUMMARY OF THE INVENTION

The present invention provides a method of preparing fluorescent polysaccharide based pH responsive compositions comprises:

a) atomization

b) encapsulation

Wherein the atomization method utilizes an encapsulation unit and the FITC-dextran fluorescent dye is encapsulated within calcium alginate microspheres

One aspect of the invention provides a pH responsive composition for the detection of pH comprising FITC-dextran encapsulated polysaccharide microspheres providing fluorescent properties. Another aspect of invention provides pH responsive composition for detection of urea comprising of FITC-dextran and Urease co-immobilized polysaccharide (Ca-alginate) microspheres.

The present invention describes compositions of polysaccharide microspheres with fluorescent properties for pH responsive detection of analytes. pH changes are the most common forms of changes occurring in the cells during a biochemical reaction and pH detection can form the basis of indirect detection of several chemical analytes. The composition of pH responsive

matrices consist of (FITC-dextran) fluorophore encapsulated calcium alginate microspheres. pH responsive fluorescence changes were detected by exposing solutions of different buffer solutions and measuring the changes using fluorescence spectroscopy. Fluorescent biodegradable microspheres formed of FITC-dextran encapsulated in alginate microspheres can form interesting tools for in vivo analyte detection.

The invention provides a single step, continuous and commercially feasible production method with a tunable control over size. pH responsive behavior was studied for development of a urea biosensor based on a co-immobilized system of urease and FITC-dextran into calcium alginate microspheres. The results indicate that urea determination using such a coimmobilized system of FITC-dextran and urease is possible in both physiological and pathological ranges of urea. Different combinations of FITC-dextran and urease loading were optimized to obtain desirable sensitivity and range of biosensing.

The method of preparation of pH sensor and a urea biosensor can be easily translated and scaled up to industrial batches. The composition developed is capable of detecting pH changes in the range (0-10 units) and urea changes from (0-50 mM) which cover the physiological and pathological levels of pH and urea concentrations. Such fluorescence based pH and urea detecting matrices can form the basis of development of point of care devices for diagnosis of diseases and health monitoring.

BRIEF DESCRIPTION OF THE DRAWINGS:

Fig. 1 provides an encapsulation unit variety J 30 and its mechanism to form alginate microspheres, showing (a) Syringe pump, (b) Urease and FITC-dextran containing alginate microspheres, (c) FITC-dextran and Urease dissolved in Na-alginate solution, (d) compressed air with a controlled pressure, (e) flow controller, (f) gas cylinder, (g) nozzle, (h) 3D conical spray of alginate, (i) FITC-dextran Urease co-immobilised Ca-alginate microspheres, (j) calcium chloride solution, (k) magnetic stirrer and (l) air flow.

Fig. 2(a), 2(b), 2(c) and 2(d) provides optical microscopic characterization of co-immobilized FITC-dextran and urease in alginate microspheres: (a) ESEM image of plain alginate

microspheres, (b) ESEM image of FITC-dextran-urease loaded alginate microspheres (c) DIC image of FITC-dextran-urease loaded alginate microspheres and (d) CLSM image of FITC-dextran-urease loaded alginate microspheres.

Fig. 2(e) and 2(f) provides a line scan of CLSM image particles of FITC-dextran-urease loaded alginate microspheres and % encapsulation efficiency of FITC-dextran (Y error bars represent the standard deviation for triplicate measurement) respectively

Fig. 3 shows the effect of FITC-dextran molecular weight on solution phase fluorescence responses in various buffer solutions of different pH.

Fig. 4(a) and 4(b) provides a) Solution phase urea sensing study (▲) (FITC-dextran and Urease both in solution phase) and b) Suspension phase urea sensing study: FITC-dextran in alginate microspheres and urease in solution phase (■) and FITC-dextran and urease both co-immobilized in alginate microspheres (♦) All measurements are mean of triplicate readings and y error bars represent the standard deviations.

Fig. 5 shows the effect of FITC-dextran loading concentration 0.25 mg/ml (■), and 0.5 mg/ml (♦) using solution phase urease catalysis. Y error bars represent the standard deviation to mean values.

Fig. 6 provides a urea sensing curve for co-immobilized system of FITC-dextran (0.5 mg/ml) and urease (1 mg/ml) in alginate microspheres, y error bars indicate standard deviation for triplicate measurements.

Fig. 7 provides commercial utilities and fields of applications of a pH Sensor and a urea biosensor.

Referring to the drawings, the embodiments of the present invention are further described. The figures are not necessarily drawn to scale, and in some instances the drawings have been exaggerated or simplified for illustrative purposes only. One of ordinary skill in the art may appreciate the many possible applications and variations of the present invention based on the following examples of possible embodiments of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of preparing fluorescent polysaccharide based pH responsive compositions comprises:

a) atomization

b) encapsulation

Wherein the atomization method utilizes an encapsulation unit and the FITC-dextran fluorescent dye is encapsulated within calcium alginate microspheres

One aspect of the invention provides a pH responsive composition for the detection of pH comprising FITC-dextran encapsulated polysaccharide microspheres providing fluorescent properties. Another aspect of invention provides pH responsive composition for detection of urea comprising of FITC-dextran and Urease co-immobilized polysaccharide (Ca-alginate) microspheres.

Water soluble polysaccharides like alginate, gelatin, chitosan can be crosslinked using some reagents like calcium salts, glutaraldehyde, or tripolyphosphates, respectively can be used as carriers for biomolecules like enzymes and fluorescent dyes to develop biosensing compositions to detect several important clinical analytes. Alginate forms the most important and widely used polysaccharide for drug delivery owing to its biocompatibility and biodegradability. The process of crosslinking or gelation can be achieved by dropping and/or atomization technique. The process of development of such microspheres can be tuned in using parameters like concentrations of alginate, CaCl2, flow rate of solution, atomizing nozzle aperture, atomizing air pressure etc. The microspheres of alginate can be formed in the sizes ranging from 10-100 μm. The optimized process has been described by Jayant et al and Joshi et al [1, 2]. Most commonly the alginate concentration is used between 1-3 % w/v in order to form spherical microspheres.

The crosslinking agent that polymerizes or gels externally the sprayed polysaccharide solutions of alginate can be a solution of divalent/trivalent metal salts. Typical metal cations useful as gelling agents for cross linking of alginates are metal cations of barium, lead, copper, strontium, cadmium, calcium, zinc, nickel and aluminium or a mixture of any of the above. The

crosslinking ability is determined by the type of the salt chosen for gelling. Carbonates, sulphate, chlorides, acetates are the most commonly used counter ions for above listed cations.

Actives like fluorescent molecules, drugs, biomolecules like proteins and enzymes can be encapsulated in the formed alginate microspheres. Fluorescent molecules for pH responsiveness include FITC and derivatives of FITC like FITC-dextran which can be measured using fluorescence spectroscopy at an excitation wavelength of 450/488 nm and emission wavelength of 520 nm. Enzymes like urease have been encapsulated in alginate microspheres to develop urea biosensors. The process involving production of microspheres includes blending of water soluble polysaccharide into water which forms the basis of polymeric material. The solution of alginate and active molecules like fluorescent dyes and enzymes were transferred to a syringe in the syringe pump (Fig 1). The said solution is sprayed using an encapsulation unit through a spray nozzle based on air flow system. The flow rate, height of the nozzle head and air pressure are varied and optimized to obtain different sizes of microspheres. Gelation occurs through this process leading to hardened microspheres

Example 1

Materials and Instruments

FITC-dextran (70kDa, 150 kDa, and 500 kDa), Alginate (Low viscosity, 2%) and Urease were purchased from Sigma-Aldrich (India), Calcium chloride and sodium carbonate was purchased from Merck (Mumbai). All chemicals were reagent grade and used as received. Encapsulation unit Variation J30 droplet generator (Nisco Engineering AG, Zurich) and syringe pump (Multi-Phaser™, model NE-1000, New Era Pump Systems, NY) were used for microspheres production.

Methods

1. Preparation of FITC-dextran encapsulated alginate microspheres

Alginate microspheres were prepared as per the procedure described by Joshi et al and Jayant et al. Briefly, FITC-dextran (0.25-1 mg/ml) was dissolved in alginate (2 % w/w) and the solution was sprayed using encapsulation unit into a well stirred $CaCl_2$ solution (4 % w/v). The solution

of FITC-dextran in alginate was sprayed at a flow rate of 20 ml/min and air pressure maintained at 75 mbar. The microspheres were centrifuged and subjected to triplicate washing cycles using deionized water. The microspheres formed were characterized using optical microscopy, SEM and CLSM. Calcium alginate microspheres prepared using a droplet generator were optimized to prepare microspheres with size 60 μ m using parameters like flow rate, concentration of sodium alginate, concentration of calcium chloride, distance of the nozzle from the surface of liquid, air pressure etc.

2. Co-Immobilization of Urease and FITC-dextran in Ca-alginate microspheres

Urease in concentrations (1-5 mg/ml) and FITC-dextran (0.25-1 mg/ml) were mixed together in sodium alginate (2 % w/w) stirred and sprayed as described earlier using the encapsulation unit. The microspheres were washed in triplicate using deionized water and then used for sensing studies of urea.

3. Characterization

The microspheres formed from alginate were characterized using optical microscopy, zeta potential, SEM and confocal laser scanning microscopy (CLSM). Encapsulation efficiency of FITC-dextran was determined using UV spectroscopy (at 455 nm and 482 nm) by estimating the amount of un-encapsulated FITC-dextran in the supernatants using a pre-determined calibration curve. Encapsulation of urease was determined by analyzing supernatants using Bradford test for protein content at 595 nm.

4. Urea biosensing studies and optimization of biosensing

Urea standard solutions were prepared in the concentration range of (0-40 mM). Co-immobilized system of urease and FITC-dextran encapsulated alginate microspheres were exposed to urea standard solutions and measured using fluorescence spectroscopy. Fluorescence spectrums were captured at excitation wavelength of 488 nm and emission wavelength of 520 nm.

Results and Discussion

1. Preparation and Co-Immobilization of FITC-dextran and Urease encapsulated alginate microspheres

Researchers report several matrices for stabilized enzyme encapsulation for biosensor development; however alginates form the most important carriers. Alginates have gained popularity in biomedical applications because of their properties of mild gelation conditions at low temperatures and without use of any toxic chemicals. Joshi et al and Jayant et al have summarized optimization studies for preparing alginate microspheres containing various biomolecules like enzymes, anti-inflammatory drugs, magnetic particles etc. Briefly, various size of microspheres can be tuned based on the instrumental parameters like flow rate, atomizing pressure, nozzle size, distance of gelation etc (Fig. 1). In general increasing atomizing pressure causes a reduction in size of microspheres although small alterations are required for maintaining sphericity of particles [*Journal of Biomedical Nanotechnology*, 3 (2007) 245-53].

The basis of formation of smaller particles using a 0.35 mm nozzle lies in the shear, friction and formation of nuclei for droplet formation after atomization using a gas generated in the droplet generator. FITC-dextran has been used by several researchers as a model high molecular weight fluorescent compound. However, very few researchers have shown the pH responsive behavior of FITC-dextran. FITC-dextran is available in different molecular weights ranging from 4kDa-2000 kDa. Different molecular weights of FITC-dextran like 70 kDa and 500 kDa were chosen for encapsulation in alginate microspheres. High molecular weight of FITCdextrans show lower possibility of leaching and hence improved stability. Urease (Mol. wt: 480 kDa) is used as urea specific biological recognition element. Urease also shows a molecular weight dependent leaching rates from crosslinked structure of alginate microspheres. In the coimmobilized form when both FITC-dextran and urease is immobilized the encapsulation efficiency is altered as the concentration of one of them is altered.

2. Characterization

Figure 2 describes the characterization of FITC-dextran-urease co-immobilized alginate microspheres using optical microscopy, SEM and CLSM. The images of optical microscopy and SEM confirmed that the microspheres formed using atomization is spherical in shape and uniform in size. The sizes of alginate microspheres can be altered by changing the instrumental

parameters for e.g. at atomizing pressures of 500 mbar particles of 10 μ m (±5 μ m) size can be formed and at 75 mbar particles having 50 μ m (±10 μ m) sizes can be formed. The particles appear spherical and well defined. The encapsulation of FITC-dextran and urease do not prove to be abrasive or structurally deforming. ESEM images indicate that the microspheres have a smooth surface and spherical morphology with porous network structure (Fig. 2a, b). Porous structure is essential for efficient analyte transfer during biosensing and will aid in reducing the response time of sensor. CLSM imaging shows that FITC-dextran molecules are uniformly distributed throughout the microspheres. The microspheres exhibited high internalization of both FITC-Dextran, 70 kDa, FITC-Dextran 500 kDa confirming the formation of co-immobilized structure (Fig. 2c, d). Line scan (Fig. 2e) and Z-Scan obtained from the CLSM scans also confirm that FITC-dextran dye is distributed uniformly in three dimensions. Jayant et al. Have described the zeta potential measurement of alginate microspheres prepared using similar method of atomization was found to be sufficiently negative to maintain their stability [*Journal of Biomedical Nanotechnology*, 3 (2007) 245-53].

Encapsulation study of FITC dextran indicated that when different concentrations of FITC-dextran were loaded in alginate microspheres the there is no significant difference in the percentage encapsulation efficiency. All the concentrations show encapsulation efficiency above 95-85 % (Fig. 2f). In presence of urease, when co-immobilization occurs, the percent encapsulation efficiency of both FITC-dextran and urease reduce to an extent of 80-85 % depending on concentration of urease used.

3. pH response studies

FITC-dextran solutions when exposed to different pH solutions ranging from 0-10 units, it was observed that a sigmoid relationship curve was obtained. FITC-dextran had minimal fluorescence intensity near highly acidic pH values (0-2 units). At pH values greater than 3 the fluorescence intensity of FITC-dextran solutions increased linearly as described in Fig. 3. The linear increase in fluorescence of FITC-dextran saturated near pH values of 8 and 9 after which there was no significant increase in fluorescence intensity values. Different molecular weights of

FITC-dextran analyzed for changes in fluorescence intensity showed no significant differences as pH values were changed. This shows that fluorescence intensity is wholly dependent of ionization of FITC and not on molecular weight of dextran attached to it.

The results also indicate that the fluorescence intensity of FITC-dextran can be linearly analyzed within pH ranges 4-8 using any molecular weight of FITC-dextran. On the basis of the fact that high molecular weight FITC-dextran like 500 kDa would be better in terms of retention of fluorescent dye within the alginate microspheres. Low molecular weight FITC-dextran will have a greater chance of getting leached out on storage. The incorporation of high molecular weight FITC-dextran can provide the necessary stability to the system.

4. Urea biosensing studies and optimization of biosensing

Joshi et al have displayed biosensing of glucose in multifunctional particles prepared by similar atomization method using glucose oxidase and an oxygen sensitive dye Rudpp. The system also showed capabilities in drug delivery and MRI imaging owing to encapsulation of magnetic nanoparticles [*Acta Biomater*, 7 (2011) 3955-63].

In this study, the fluorescence of the solutions was measured as a function of time until the signal reached steady state. Biosensor performance is based on alterations in fluorescence intensity of pH responsive FITC-dextran dye. Urease catalyzes the conversion of urea to produce ammonium and OH- ions. OH- ions so produced react with FITC and ionize it. Ionization of fluorescent dye causes an increase in fluorescence intensity. In order to reduce inter-sample and instrumental variations I/Io are plotted against the concentration of urea.

Solution phase sensing studies involved use of FITC-dextran and urease both in solutions showed that a linear response was observed in from sub-milimolar concentrations to about 1.5 mM with a regression coefficient of 0.894 (Fig 4a). The physiological levels of urea in serum has been reported to be 2-8 mM, hence in solution phase the urea detection is not sufficient. When FITC-dextran was encapsulated in alginate microspheres at concentrations from 0-1 mg/ml, the urea detection range was improved till 7 mM with a regression coefficient 0.986. Conversely, when both FITC-dextran and urease were co-immobilized in alginate microspheres the urea

detection range reduced till 5 mM at a good regression coefficient of 0.9847 as described in fig.4b. The reduced range after co-immobilization could be explained by the inactivation or reduction of part of activity of enzyme in presence of FITC-dextran. In order to understand the affecting parameters for urea detection different optimization steps were carried out which include different dye loading concentrations (0.25, 0.5, 0.75 and 1 mg/ml) and different enzyme loading concentrations (0.5, 1, 4 mg/ml). To understand effect of FITC-dextran loading different loading concentration of FITC-dextran were evaluated using a urease solution outside alginate microspheres.

The results indicated that when a concentration of FITC-dextran was increased from 0.25 mg/ml to 0.5 mg/ml the range could be increased slightly ie, from 4 mM to 6 mM following a similar good regression coefficients (Fig 5). The sensitivity value for the corresponding urea response curve was found to be 44.8 %/mM in the calibration range which was calculated from change in intensity ratio during the reaction. The linearity was established within the range of 0-50mM (Fig.6). Additionally it was revealed that decreasing the sample volume increased the analytical range of urea determination. There were no significant differences in urea sensing upon increase in concentration of enzyme and fluorescent dyes. As observed in the pH response curves of both molecular weights of FITC-dextran the fluorescence intensity is linear only till pH 8-9.

In order to obtain higher urea concentration determined the volume of solution fed to a set of microsphere particles need to be reduced. These results are promising as further improvement in sensitivity of glucose detection can be done by optimizing microsphere size, enzyme concentration, and permeability to urea. Further the method provides with capability of detecting any analyte (based on pH) just by changing the enzyme in the matrix.

Solution proposed by the present invention

Urea is an important marker for diseases related to kidney and liver conditions. With the advent of developments in biosensors, a urea biosensor appears more feasible to help patients monitor the urea levels using a point of care device. The proposed invention can be transformed into an important sensing assay for use as a urea biosensor. The FITC-dextran loaded microspheres act as a pH sensor and when urease is co-immobilized with FITC-dextran in the alginate microspheres it will act as a urea biosensor. Both pH and urea biosensors can be

beneficial markers for detection and monitoring of kidney diseases. It has been developed based upon measurement of the pH change, using an appropriate pH sensitive dye, produced in the aqueous environment by the products of the urease catalyzed hydrolysis of urea. The microsphere matrix has the capability to measure pH in the range of 0-10 units and urea concentration within the range of 0-50 mM. The biosensor assay gives a linear range in lower range (below 2 mM) as well as higher range till 50 mM of urea concentration.

The working principle of the sensor is based upon measurement of the pH change produced in an aqueous environment by the products of the enzyme-catalyzed hydrolysis of urea as shown in the following reaction (Eq. 1).

$$NH_2CONH_2 + 3H_2O \rightarrow 2NH_4^+ + HCO^{3-} + OH^- \quad \text{.....(1)}$$

Changes in the concentration of products i.e. ammonium, bicarbonate and hydroxide ions remaining in equilibrium are proportional to the concentration of urea in the sample solution. The solution was researched in order to develop a pH sensor and a urea sensor based on this reaction using urease as a catalyst. The pH reaction is coupled to a fluorescent dye (FITC-dextran) which is responsive to changes in concentration of OH- ions. The changes in intensity of fluorescence of FITC-dextran are recorded and quantified as a measure of changes in urea concentrations.

Commercial applications for the proposed technology

Urea biosensor can find several applications in clinical analyte determination in dialysate monitoring, blood urea management, diagnosis of diseases related to kidney, environmental monitoring, analytical devices to measure pH and urea. Apart from these applications, a urea biosensor can be used for E. coli detection and heavy metal detection owing to inhibition of urease in response to heavy metals. The global market for Biosensors in 2012 was US$ 12 billion and is estimated to touch US$16.8 billion by 2018 at an annual growth rate of 11% [11]. The dialysis industry remains a robust, multi-billion business that has managed to fuel its growth engine in the past five years. The other potential markets for urea biosensors are as shown in Fig.7. The stable customer base explains how the dialysis industry is non cyclical in nature and is only minimally affected by the vagaries of economic downturns. The US market for ESRD dialysis devices, including hemodialysis and peritoneal dialysis products such as dialyzers,

hemodialysis machines, access devices, transfer sets, cyclers, and catheters will continuously grow. World dialysis market was around $75 Billion in 2011 [http://reports.fmc-ag.html].

We claim:

1. A method of preparing fluorescent polysaccharide based pH responsive compositions comprises:
   a) atomization
   b) encapsulation
2. According to the method of claim 1, the atomization method utilizes an encapsulation unit
3. According to the method of claim 1, the FITC-dextran fluorescent dye is encapsulated within calcium alginate microspheres.
4. A method of preparing pH responsive composition for detection of urea comprising of FITC-dextran and Urease co-immobilized polysaccharide (Ca-alginate) microspheres.
5. According to the method of claim 4, the composition of pH responsive matrices consist of (FITC-dextran) fluorophore encapsulated calcium alginate microspheres.
6. According to the method of any of claim 1 and 4, the pH responsive fluorescence changes were detected by exposing solutions of different buffer solutions and measuring the changes using fluorescence spectroscopy.
7. According to the method of any of claim 1 and 4, different combinations of FITC-dextran and urease loading were optimized to obtain desirable sensitivity and range of biosensing.
8. According to the method of any of claim 1 and 4, water soluble polysaccharides but not limiting to like alginate, gelatin, chitosan are used for crosslinking.
9. According to the method of any of claim 1 and 4, carriers for biomolecules are selected from but not limited to reagents like calcium salts, glutaraldehyde, or tripolyphosphates.
10. According to the method of any of claim 1 and 4, gelling agents for cross linking of alginates are selected from but not limited to metal cations such as metal cations of barium, lead, copper, strontium, cadmium, calcium, zinc, nickel and aluminium or a mixture of any of the above.
11. According to the method of any of claim 1 and 4, counter ions are selected from but not limited to carbonates, sulphate, chlorides, acetates
12. According to the method of any of claim 1 and 4, the composition developed is capable of detecting pH changes in the range (0-10 units) and urea changes from (0-50 mM) which cover the physiological and pathological levels of pH and urea concentrations.

13. According to the method of claim 12, wherein the composition can be transformed into an important sensing assay for use as a urea biosensor and used in the development of point of care devices for diagnosis of diseases and health monitoring of disease.
14. According to the method of claim 13, the sensor is based upon measurement of the pH change produced in an aqueous environment by the products of the enzyme-catalyzed hydrolysis of urea.
15. According to the method of any of claim 1 and 4, production of microspheres includes: blending of water soluble polysaccharide into water which forms the basis of polymeric material; transferring a solution of alginate and active molecules like fluorescent dyes and enzymes to a syringe in the syringe pump.
16. According to the method of claim 15, wherein the said solution is sprayed using an encapsulation unit through a spray nozzle based on air flow system.
17. According to the method of claim 16, wherein the flow rate, height of the nozzle head and air pressure are varied and optimized to obtain different sizes of microspheres
18. According to the method of claim 17, wherein gelation occurs through this process leads to hardened microspheres.

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