

RAPID BIOASSAY FOR PESTICIDE RESIDUE USING SOL GEL MATRIX

Technical Field of the Invention

According to the present invention, a method is provided to encapsulate Acetyl Cholinesterase (Ache) by forming a silica sol from a solution of silicon oxide in water, adjusting the pH to a pH value less than 7 to stabilize the silica sol, forming a silica sol matrix solution containing Acetyl Cholinesterase. The invention provides technique for biological capture medium wherein the enzyme remain stable and active for long period of time if stored at 4 deg. In the disposable methacrylate cuvettes. The technology offers a disposable type of prepared enzyme assay in disposable methacrylate cuvettes. The encapsulated enzyme was found to react with pesticides namely Organophosphate and Carbamate group which are widely used for vegetables control against pests. The mode of action of this interaction in the sol gel is inhibition activity where in the colour reaction form by a substrate (ACTI) together with DTNB with the enzyme is yellowish in colour. The colour was found to be developed in the matrix gel i.e. in the gel cavity. By minor shaking of this cuvet containing solgel the colour is released to the free solution which can be seen by naked eye. However for accurate measurement of the inhibition activity, the colour formation was measured for their absorbance using calorimetric spectrophotometer. The percentage different in colour change compare to a control i.e. solution with no pesticide was used as a basis for the calculation. The threshold value for this percentage inhibition was set to a minimum level as a basis to determine the acceptance level of pesticide content in the sample.

Background of the Invention

The invention relates to a sol-gel method for encapsulation of AchE use for bioassay development. Development of new materials that can improve the bioassay capabilities has improved dramatically. One of the most challenging aspects in development of these biological support matrixes is the immobilisation and integration of biological molecules in the host matrix and retaining the functionality of the biomolecules. A number of techniques such as physical

adsorption, covalent attachment, entrapment and encapsulation in polymer as well in inorganic matrices have been explored over the years to achieve a reproducible and robust immobilisation technique that preserves the activity of the biological molecule.

5 Silica host matrices, made by the sol-gel process have emerged as a promising platform for encapsulation of biomolecules such as enzymes, antibodies and cells. Enzymes find a more stable environment upon encapsulation in silica host because the polymeric framework grows around the biomolecule, creating cage and thus protecting the enzyme from aggregation and unfolding. These sol-gel
10 matrices are chemically inert, thermally stable, hydrophilic and transparent, enabling spectroscopic monitoring of the entrapped sample. Microencapsulation in the pores of the sol-gel matrix does not decrease the affinity and activity of biomolecules because the enzyme is not really bound to the matrix. The purpose of this invention is to develop a sol-gel encapsulated housefly
15 acetylcholinesterase as a biosensing material that will be used in the development of bioassay for screening of pesticide residues in vegetables.

Summary of the Invention

Accordingly, it is the primary object of the present invention to provide a method for making a porous, transparent sol-gel matrix containing a biological material
20 encapsulated therein.

It is another object of the present invention to provide a method for the quantitative or qualitative detection of a test substance that reacts with or whose reaction is catalyzed by an active biological material, wherein said biological material is encapsulated within a sol-gel matrix, and wherein said sol-gel matrix
25 is prepared by an alcohol-free method.

It is yet another object of the present invention to provide a method for storing a biological material in a porous, transparent sol-gel matrix, wherein withstanding exposure to adverse conditions, about 80% of the biological activity of the material is retained for at least 6 months.

These and other objects of the present invention are accomplished by providing,

A method of making a porous, transparent sol-gel matrix containing a biological material encapsulated therein, comprising:

- a) forming a single phase sol by mixing a metal alkoxide in a non-alcoholic medium comprising water and an acid catalyst and stirring the mixture until a clear solution is obtained;
- b) adding a buffering agent to raise the pH of the mixture obtained in step (a) to a pH of between 5-7;
- c) adding a biological material to the buffered sol in step (b);
- d) forming a sol-gel and allowing the said sol-gel to age; and
- e) allowing a portion of the water in the sol-gel to evaporate so that the volume of the product formed in step (d) is decreased and the biological material is encapsulated in a monolith of the sol-gel having a reduced volume.

A method for the quantitative or qualitative detection of a test substance that reacts with or whose reaction is catalyzed by an active biological material, wherein said biological material is encapsulated within a sol-gel matrix, and wherein said sol-gel matrix is prepared by an alcohol-free method, said quantitative or qualitative method comprising:

- a) preparing the sol-gel matrix comprising said active biological material encapsulated within a porous, transparent matrix prepared by an alcohol-free sol-gel method;
- b) bringing the said biological material-containing sol-gel matrix into contact with a gas or aqueous solution comprising the test substance; and
- c) quantitatively or qualitatively detecting, observing or measuring the change in one or more characteristics in the biological material encapsulated within the sol-gel matrix.

A method of storing a biological material in a porous, transparent sol-gel matrix, wherein withstanding exposure to adverse conditions, about 80% of the biological activity of the material is retained for at least 6 months, comprising:

- a) forming a single phase sol by mixing a metal alkoxide in a non-alcoholic

medium comprising water and an acid catalyst and stirring the mixture until a clear solution is obtained;

- b) adding a buffering agent to raise the pH of the mixture obtained in step (a) to a pH of between 5-7;
- 5 c) adding a biological material to the buffered sol in step (b);
- d) forming a sol-gel and allowing the said sol-gel to age; and
- e) allowing a portion of the water in the sol-gel to evaporate so that the volume of the product formed in step (d) is decreased and the biological material is encapsulated in a monolith of sol-gel having a reduced volume; and
- 10 f) storing the gel of step (e) and the biological material encapsulated therein.

Detailed Description of the Invention

The present invention has been accomplished for circumstances described above. Its objective is to provide a rapid bioassay for determining pesticide residues in samples, such as vegetables and fruits. To attain the above objective, the

15 inventors conducted in-depth studies, and obtained the results based on the interaction of the enzyme with the pesticides at various buffer concentration and pH values. The mixture ratio of sol gel and buffer was extensively studied and the optimum mixture was obtained in the experiment. A new biosensing material based on sol-gel encapsulation with enzyme was developed for rapid detection of

20 organophosphate pesticide residues. Three types of organophosphate (OP) with different toxicity i.e. dichlorvos (class I-highly toxic and hazardous compound), chlorpyrifos and fenitrothion (class II-slightly toxic compounds) were used to study the effectiveness of the sol-gel immobilisation. Enzymatic inhibition activities showed that the response of the biosensing material to these Ops were

25 highly reproducible. The enzyme-doped sol-gel monolith was found to be stable for three month under storage at 4 deg. In addition, the gel can be mass produced thus making it cheap enough to be disposed after a single use. Comparison data on spectrophotometer methods indicated that the methods showed similar trend in results when tested on spiked samples with pesticide. The newly developed

30 material i.e. sol-gel encapsulated with AChE can be used as a biological reagent for fast detection of organophosphate pesticide (OP).

Our current bioassay can utilise this biosensing material as a biorecognition element. The encapsulation was carried out at room temperature and phosphate buffer was used to minimise the denaturation of enzymes. Three types of OP with different toxicity i.e. dichlorvos (category I-highly toxic and hazardous compound), chlorpyrifos and fenitrothion (category II-slightly toxic compounds) were used in this study. The calibration plots for dichlorvos, chlorpyrifos and fenitrothion with dynamic range between 0.1-1.0 ppm, 20-100 ppm and 100-1000 ppm respectively were obtained. Competitive inhibition was observed when halves of maximum inhibition (I_{50}) of these Ops were reacted with various concentrations of substrate, acetylthiocholine iodide (ATCI). Enzymatic inhibition showed that the response of the biosensing material to these Ops were highly reproducible. The lower percentage of enzyme leaching (20%) shows that the microencapsulation process is able to entrap enzyme in the sol-gel matrix. The enzyme-doped sol-gel monolith was found to be stable for three month under storage at 4 deg. In addition, the gel can be mass produced thus making it disposable after a single use. Recovery data were collected for dichlorvos on two varieties of vegetables, amaranth or chinese spinach (*Amaranthus viridis*) and convolvulus (*Ipomoea aquatica*). The percentage recovery of approximately 80% for spiked samples with average RSD of 2.44% shows that this newly developed biosensing material can be used as a bioreceptor for the biosensor device.

Preparation of sol-gel

The sol-gels were prepared according to that described by Ellerby et al. [3] employing a two-step method. The acid catalyzed silica sol was first prepared by mixing 4.5 ml of TMOS, 1.4ml of H_2O and 0.1 ml HCL in a glass-vial according to Kumar [4] with a change in the precursor used. The mixture was stirred until a clear solution was obtained. The vial was sealed and polymerization took place for 24 hours to obtain a suitable silicate polymer gel.

Chemicals and reagents

Acetylcholinesterase (1.25 U mg^{-1}) from housefly (HFACH_E) was purchased from Taiwan Agricultural Research Institute (TARI). Acetylthiocholine iodide

(ATCI) and 5,5' -dithio-bis (2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co. The organophosphorus (OP) pesticides, dichlorvos (99%) and chlorpyrifos (99%) were purchased from Riedel de Haen (Seelze, Germany). Stock standards of 100 $\mu\text{g ml}^{-1}$ concentrations were prepared in methanol and stored at 4°C. Tetramethyl orthosilicate (TMOS) was purchased from Tokyo Kasei, TCI (grade 99%). Methanol, hydrochloric acid (HCl) and other chemicals used were from Merck. A phosphate buffer solution of pH 8 and 0.1 M was prepared. The disposable methacrylate cuvettes were obtained from Dispolab-Kartell, 1.5 ml.

10 **Enzyme Entrapment in the Sol-Gel Matrices**

A mixture of 70 μl of buffer, 20 μl of HFACHe (0.5 U ml^{-1}) and 30 μl of sol were put into a disposable cuvette and the sol-gel solidified within seconds. The enzyme was encapsulated completely within the growing network. The cuvettes were sealed with parafilm to prevent cracking, through rapid evaporation of the gels during ageing. Complete gelation took approximately 15 min after which time, the gel was stored at 4°C for aging.

Leaching Test

Two different cuvettes were used to add 20 μl of HFACHe, and 20 μl of sol respectively. The third cuvette was added with 30 μl sol, 70 μl of buffer and 20 μl of AchE and immobilisation was allowed for one minute before it was measured. One ml of buffer was added to each cuvette and the optical density of protein was measured at wavelength 280 nm using spectrophotometer.

Recovery Test

Vegetable sample (spinach) was chopped into small pieces and a subsample (50 g) was weighed and placed in a blending jar. It was homogenised with 50 μl of 1000 ppm dichlorvos for one hour. After incubation, a volume of 50 ml methanol was added and the mixture was blended for 5 minutes. The extract was filtered through a filter paper and the filtrate was collected. For the control, similar procedures were carried out except the homogenization with dichlorvos. The analytical procedure for the enzymatic inhibition was similar to work

reported by Salmah et al. [5]. A volume of 50 μl filtrate was incubated in a cuvette containing AchE-doped sol-gel for 10 min. The absorbance was determined spectrophotometrically after 5 min of substrate addition.

Characterisation of Enzyme-Doped Sol-Gel Monolith

- 5 TMOS-derived sol-gel monolith was optically transparent and porous and hence suitable for the encapsulation of AchE. Previous results of the optimised working conditions such as composition ratio of (buffer + enzyme) to sol was 3:1 enzyme quantity of 0.5 U and working substrate concentration of $5.0 \times 10^{-3}\text{M}$ were used for the characterisation of the biosensing material [6]. Leaching test
- 10 was performed successfully prior to the characterisation study where leaching percentage of the enzyme-doped sol-gel monolith compared to free enzyme are shown in table 1.0. The optical density of protein (280 nm showed that 80% of the encapsulated AchE was retained in the sol-gel matrices with a gelation period of one minute. According to Bhatia and Brinker [1], the pores of the enzyme-
- 15 doped silica matrix should be small enough to prevent leaching of encapsulated macromolecules but large enough to allow unrestricted transport of buffer ions, molecules of substrate and analyte.

References:

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In the foregoing description, the present invention has been specifically disclosed by referring to some examples thereof. However, it should be appreciated that various modifications and variations can easily be made by those skilled in the art without departing from the spirit of the invention. It is , thus, intended to include all such modifications and variations within the wide scope of the present invention defined by the appended claims.

Claims:

- 1) A method of making a porous, transparent sol-gel matrix containing a biological material encapsulated therein, comprising:
 - a) forming a single phase sol by mixing a metal alkoxide in a non-alcoholic medium comprising water and an acid catalyst and stirring the mixture until a clear solution is obtained;
 - b) adding a buffering agent to raise the pH of the mixture obtained in step (a) to a pH of between 5-7;
 - c) adding a biological material to the buffered sol in step (b);
 - d) forming a sol-gel and allowing the said sol-gel to age; and
 - e) allowing a portion of the water in the sol-gel to evaporate so that the volume of the product formed in step (d) is decreased and the biological material is encapsulated in a monolith of the sol-gel having a reduced volume.
- 2) The method of Claim 1, wherein the metal alkoxide is tetramethyl orthosilicate (TMOS), tetraethyl orthosilicate (TEOS), or any other suitable material.
- 3) The method of Claim 1, wherein the acid catalyst is hydrochloric acid (HCl) or any other suitable catalyst.
- 4) The method of Claim 1, wherein the optimized ratio of buffer plus enzyme to sol-gel is 3:1
- 5) The method of Claim 1, wherein the size of the sol particle is selected to produce a pore size when the sol-gel is dried, which is essentially the same size as the size of the molecule of the encapsulated biological material.
- 6) The method of Claim 1, wherein the biological material is selected from the group consisting of RNA, DNA, active proteins, active fragments of DNA, RNA, or proteins, and cells or tissues.

- 7) The method of Claim 6, wherein the biological material is any active protein or active fragment thereof.
- 8) The method of Claim 7, wherein the protein or active fragment thereof is an enzyme or active compound selected from the group consisting of acetylcholinesterase, or any enzyme or active compound.
- 9) A method for the quantitative or qualitative detection of a test substance that reacts with or whose reaction is catalyzed by an active biological material, wherein said biological material is encapsulated within a sol-gel matrix, and wherein said sol-gel matrix is prepared by an alcohol-free method, said quantitative or qualitative method comprising:
- a) preparing the sol-gel matrix comprising said active biological material encapsulated within a porous, transparent matrix prepared by an alcohol-free sol-gel method;
 - b) bringing the said biological material-containing sol-gel matrix into contact with a gas or aqueous solution comprising the test substance; and
 - c) quantitatively or qualitatively detecting, observing or measuring the change in one or more characteristics in the biological material encapsulated within the sol-gel matrix.
- 10) A method for the quantitative or qualitative detection of pesticide according to any one of the preceding claims.
- 11) The method of Claim 10, wherein the pesticide is an organophosphate selected from the group consisting of dichlorvos, chlorpyrifos, fenitrothion, or any other organophosphate.
- 12) A method of storing a biological material in a porous, transparent sol-gel matrix, wherein withstanding exposure to adverse conditions, about 80% of the biological activity of the material is retained for at least 6 months, comprising:

- a) forming a single phase sol by mixing a metal alkoxide in a non-alcoholic medium comprising water and an acid catalyst and stirring the mixture until a clear solution is obtained;
 - b) adding a buffering agent to raise the pH of the mixture obtained in step (a) to a pH of between 5-7;
 - c) adding a biological material to the buffered sol in step (b);
 - d) forming a sol-gel and allowing the said sol-gel to age; and
 - e) allowing a portion of the water in the sol-gel to evaporate so that the volume of the product formed in step (d) is decreased and the biological material is encapsulated in a monolith of the sol-gel having a reduced volume; and
 - f) storing the gel of step (e) and the biological material encapsulated therein.
- 13) The method of storing a biologically active material in a porous, transparent sol-gel matrix according to Claim 12, wherein the biological material is an active protein or active protein fragment, wherein the metal alkoxide comprises tetramethyl orthosilicate (TMOS), tetraethyl orthosilicate (TEOS), or any other suitable material, and wherein withstanding exposure to adverse conditions, about 80% of the biological activity of the protein or protein fragment is retained for at least 6 months.
- 14) The active biological material which has been stored according to Claim 12 or 13 for at least 6 months, but wherein about 80% of the biological activity of the material has been retained.
- 15) The method of Claim 13 or 14, wherein the biological material, active protein or active protein fragment is acetylcholinesterase or any other enzyme or active compound.