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Fabrication and Characterization of Sol-Gel Based Nanoparticles for Drug Delivery

Reeta Yadav

Graduate Center, City University of New York

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FABRICATION AND CHARACTERIZATION
OF
SOL-GEL BASED NANOPARTICLES
FOR DRUG DELIVERY

by

Reeta Yadav

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy. City University of New York

2014
This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

Uri Samuni

Date

Uri Samuni

Chair of Examining Committee

Edward Kennelly

Date

Executive Officer

George John

Hiroshi Matsui

Sanjai Kumar

Glendon Dale McLachlan

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK
Abstract

FABRICATION AND CHARACTERIZATION OF SOL-GEL BASED NANOPARTICLES FOR DRUG DELIVERY

By

Reeta Yadav

Adviser: Professor Uri Samuni

Nanogels are cross linked polymeric sol-gel based nanoparticles that offer an interior network for incorporation and protection of biomolecules, exhibiting unique advantages for polymer based delivery systems. We have successfully synthesized stable sol-gel nanoparticles by means of [a] silicification reactions using cationic peptides like polylysine as gelating agents, and [b] lyophilization of sol-gels. Macromolecules such as Hemoglobin and Glucose Oxidase and small molecules such as Sodium Nitroprusside (SNP) and antibiotics were encapsulated within the nanogels. We have used transmission electron microscopy, dynamic light scattering, zeta potential analysis, and spectroscopy to perform a physicochemical characterization of the nanogels resulting from the two approaches. Our studies have indicated that the nanogel encapsulated proteins and small molecules remain intact, stable and functional.

A Hydrogen Peroxide (H₂O₂) and Nitric Oxide (NO) generating drug carrier was synthesized using these nanogels and the effect of generation of H₂O₂ from Glucose Oxidase encapsulated nanogels and NO from SNP encapsulated nanogels was tested on E.coli. The results show that the nanoparticles exert antimicrobial activity against E.Coli, in addition NO generating nanogels potentiated H₂O₂ generating nanogels induced killing. These data suggest that these NO and H₂O₂ releasing nanogels have the potential to serve as a novel class of
antimicrobials for the treatment of multidrug resistant bacteria. The unique properties of these protein/drug incorporated nanogels raise the prospect of fine tailoring to specific applications such as drug delivery and bio imaging.
ACKNOWLEDGEMENTS

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CHAPTER 1
INTRODUCTION

Rapid developments in the pharmaceutical drug discovery are resulting in an increasing number of novel therapeutic drugs for the treatment of a variety of diseases. However, the main problems associated with systemic drug administration for most diseases still remain, which includes the even bio distribution of administered pharmaceuticals throughout the body, the lack of drug specific affinity toward a pathological site, nonspecific toxicity and other side effects resulting from high doses. There has been a rapid growth of interest in the use of nanotechnology and nanoparticles. Nanoparticle based drug delivery and drug targeting systems aim to minimize drug degradation and inactivation upon administration, prevent undesirable side effects and increase drug bioavailability. The use of nanoparticles as pharmaceutical carriers to enhance the in vivo efficiency of many drugs has been well established over the past decade, in both pharmaceutical research and the clinical setting [1].

The key parameters in the design and formulation of carrier systems are preparation and entrapment method, size, stability and degradation characteristics of the bioactive-carrier complex as well as release kinetics of the entrapped material. In general biodegradable and bio absorbable carriers are preferred so that they would degrade inside the body by hydrolysis or by enzymatic reactions, without producing any toxic products. Over the years numerous techniques have been developed for the preparation of the carrier systems and entrapment of bioactives to these carriers [2]. However, most of these methods are not suitable for entrapment of sensitive substances because of their exposure either to mechanical stresses (e.g. high-shear homogenization, sonication, high pressures), potentially harmful chemicals (e.g. volatile organic solvents, detergents), or low/high values of pH during the preparation. Many of these preparation techniques require the application of harmful volatile solvents (e.g. chloroform, ether, methanol,
acetone) to solubilize the ingredients. Such solvents not only affect the chemical structure of the entrapped substance but also may remain in the final formulation and contribute to toxicity and influence the stability of the carrier systems. In general, residual organic solvents have no therapeutic benefits, but may be hazardous to human and animal health as well as the environment [2-4]. In addition to the above mentioned disadvantages, use of volatile organic solvents necessitates performance of two further steps in the synthesis of the carrier systems: i) removal of these solvents, and ii) assessment of the level of residual organic solvents in the formulations.

Nanogels are a recent addition to the list of known physical nanocarriers, which have found applications as drug delivery systems. The term ‘nanogels’ defines dispersions of hydrogel particles formed by physically or chemically cross-linked polymer networks of nanometric dimensions. Hydrophilic particles with high dispersion stability can be produced by loading biological agents and drugs into nanogels via a spontaneous process including interactions between the agent and the polymer matrix. Studies have shown that nanogels were able to physically protect biological molecules from degradation in vivo and have been investigated for different types of active molecules, ranging from small drugs to bio macromolecules [3]. Nanogels show excellent potential as drug delivery carriers because of their high loading capacity, high stability and responsiveness to environmental factors, such as ionic strength, pH, and temperature that are unprecedented for common pharmaceutical nanocarriers [1].

Many approaches have been used for the preparation of nanogels, most of which can be divided into 1) physical self-assembly of interactive polymers; 2) polymerization of monomers in a homogeneous phase or in a micro or nanoscale heterogeneous environment; 3) crosslinking of preformed polymers; and 4) template assisted nanofabrication of nanogel particles [3]. A method
of great interest is the sol-gel process, which is a wet-chemical technique widely used in the fields of materials science and ceramic engineering. Sol-gel materials are prepared by generation of colloidal suspensions (“sols”) which are subsequently converted to viscous gels and thence to solid materials. Typical precursors for this gel are metal alkoxides, which undergo various forms of hydrolysis and polycondensation reactions. Lately there has been considerable increase in sol-gel synthesized materials for biological applications as they offer many advantages compared to other conventional methods. The temperatures required for most stages are close to room temperature thus minimizing any thermal degradation of both the material itself and any entrapped species, leading to high purity and higher yield. Due to the mild temperature and pH conditions used, pH sensitive organic species (e.g. Dyes) and even biological species including enzymes and whole cells can be entrapped and still retain their functions. Another reason why this technique is valuable is because appropriate chemical modification of the precursors, can give control over the rates of hydrolysis and condensation and thus change colloid particle size and the pore size, porosity and pore wall chemistry of the final material. Also it is possible to do covalent attachment of organic and biological species to porous silicate glass structures using functionalized precursors [4,5]. All these properties of sol-gel are very useful for an optimal nanotherapy because it is important that the therapeutic molecule is protected from degradation and reach its targeted intracellular locations without interactions with non-target cells.

Interestingly, certain organisms such as diatoms (Figure 1) have developed mechanisms for generating intricate structures based on silica. This formation of in vivo silica based structures has been studied in depth and it is known that biosilicification occurs under mild physicochemical conditions. A recent work published in Science by Kroger et al.[6] has shown that silica precipitating proteins (silaffin proteins) such as R5 (Figure 2) from the diatom
*Cylindrotheca fusiformis* can catalyze the precipitation of silica within seconds when added to a solution of silicic acid with a resulting material of fused spherical silica particles with an average diameter of 500nm [7-9]. Entrapment and enzyme immobilization studies done with butyrylcholinesterase and catalase have revealed that biosilica not only provides an excellent immobilization and matrix support but also retains 90% of the enzyme activity [8].

Thus biosilicification reactions catalyzed by silica precipitating peptides can be used as a new route for the entrapment of an exogenously added enzyme or drug in the silica matrix. Use of Poly-L-Lysine (PLL) (Figure 2) which is also a silica precipitating peptide has the additional advantages of utilizing the chemical properties of lysine for functionalization of the nanoparticles and selective binding of bioactive agents. Naik et al. have shown that the formation of biosilica structures can be controlled by manipulation of the physical reaction environment; they showed several silica nanomorphologies ranging from common spheres to highly organized and complex fibrillar morphologies, catalyzed by the R5 peptide under varied conditions [9].

![Figure 1](image_url)

THE SOL- GEL PROCESS

The term sol-gel refers to a process in which solid nanoparticles dispersed in a liquid (a sol) agglomerate together to form a continuous three-dimensional network extending throughout the liquid (a gel). The sol-gel process is a method for producing solid materials from small molecules. The method is used for the fabrication of metal oxides, especially the oxides of silicon and titanium. Typical precursors are metal alkoxides and metal chlorides, which undergo hydrolysis and polycondensation reactions to form a colloid. The basic structure or morphology of the solid phase can range anywhere from discrete colloidal particles to continuous chain-like polymer networks [5].

In either case (discrete particles or continuous polymer network) the sol leads to the formation of an inorganic network containing a liquid phase (gel). Formation of a metal oxide involves connecting the metal centers with oxo (M-O-M) or hydroxo (M-OH-M) bridges, therefore generating metal-oxo or metal-hydroxo polymers in solution. A drying process serves to remove the liquid phase from the gel, yielding a micro-porous amorphous glass.

A well-studied alkoxide is tetramethyl orthosilicate (TMOS). The chemical formula for TMOS is given by Si(OCH₃)₄, or Si(OR)₄, where the alkyl group R = CH₃. Alkoxides are ideal chemical
precursors for sol-gel synthesis because they react readily with water. In a hydrolysis reaction, a hydroxyl ion becomes attached to the silicon atom as follows:

\[
\text{Si(OR)}_4 + \text{H}_2\text{O} \rightarrow \text{HO-Si(OR)}_3 + \text{R-OH}
\]

Depending on the amount of water and catalyst present, hydrolysis may proceed to completion to silica:

\[
\text{Si(OR)}_4 + 2 \text{H}_2\text{O} \rightarrow \text{SiO}_2 + 4 \text{R-OH}
\]

Complete hydrolysis requires a significant excess of water and catalysts such as acetic acid or hydrochloric acid. Intermediate species include \([(\text{OR})_2-\text{Si-(OH)}_2]\) or \([(\text{OR})_3-\text{Si-(OH)}]\), resulting from partial hydrolysis reactions. Two partially hydrolyzed monomers would result in early intermediates linked via a siloxane \([\text{Si-O-Si}]\) bond:

\[
(\text{OR})_3 - \text{Si-OH} + \text{HO-Si-(OR)}_3 \rightarrow [(\text{OR})_3\text{Si-O-Si(OR)}_3] + \text{H-O-H}
\]

or

\[
(\text{OR})_3 - \text{Si-OR} + \text{HO-Si-(OR)}_3 \rightarrow [(\text{OR})_3\text{Si-O-Si(OR)}_3] + \text{R-OH}
\]

Thus, polymerization is associated with the formation of a 1, 2, or 3-dimensional network of siloxane \([\text{Si-O-Si}]\) bonds accompanied by the production of H-O-H and R-O-H species (Figure 3).

Condensation liberates a small molecule, such as water or alcohol. This type of reaction can continue to build larger and larger silicon-containing molecules by the process of polymerization. The hydrolysis and polycondensation reactions initiate at numerous sites within
the TMOS + H₂O solution as mixing occurs. When sufficient interconnected Si-O-Si bonds are formed in a region, they cooperatively form colloidal (submicrometer) particles or a sol. The size of the sol particles and the cross-linking within the particles (i.e., density) depend upon the pH and R ratio (R = [H₂O]/[Si(OR)₄]) Polymerization of silicon alkoxide, for instance, can lead to complex branching of the polymer, because a fully hydrolyzed monomer Si(OH)₄ is tetrafunctional. Alternatively, under certain conditions such as low water concentration fewer than 4 of the OR or OH groups (ligands) will be capable of condensation, so relatively little branching will occur. The mechanisms of hydrolysis and condensation, and the factors that direct the structure toward linear or branched structures are the most critical issues of sol-gel science and technology. This reaction is favored in both basic and acidic conditions [10].

**Figure 3.** Simplified representation of condensation of TMOS in the sol-gel process. (Top) Tetra methyl ortho silicate monomer and (bottom) the sol-gel matrix.
Since the sol is a low-viscosity liquid, it can be cast into a mold. The mold must be selected to avoid adhesion of the gel. With time the colloidal particles and condensed silica species link together to become a three-dimensional network. The physical characteristics of the gel network depend greatly upon the size of particles and extent of cross-linking prior to gelation. At gelation, the viscosity increases sharply, and a solid object results in the shape of the mold. Aging of a gel involves maintaining the cast object for a period of time, hours to days, completely immersed in liquid. During aging, polycondensation continues along with localized solution and re-precipitation of the gel network, which increases the thickness of interparticle necks and decreases the porosity. The strength of the gel thereby increases with aging. An aged gel must develop sufficient strength to resist cracking during drying [4,5,10].

Although several studies have demonstrated the synthesis of silicate particles using synthetic peptides and polyamines, there is not much work done on physicochemical characterization and optimization of these particles which is much needed before they can be used for therapeutic drug delivery. Our aim with our experiments was to extend these studies and find optimum synthetic conditions to produce stable sol-gel based nanoparticles which can be fabricated and characterized for drug delivery and a wide number of other applications. We report here use of two different sol-gel based techniques to synthesize nanogels (i) Sol-gel lyophilization technique and (ii) a biomimetic polycationic peptide technique. We also performed a physicochemical characterization of the resulting nanogels from the two techniques and report future applications.
CHAPTER 2

SYNTHESIS OF SOL-GEL BASED NANOPARTICLES-
DIFFERENT APPROACHES

Two different approaches were used for synthesis of sol-gel based nanoparticles, the Polycationic peptide protocol and the Lyophilization protocol. Both approaches start out with the hydrolysis and condensation of a common precursor alkoxide, Tetramethyl ortho silicate (TMOS) (Figure 4). Once the colloidal solution (sol) of TMOS is formed, (i) nanoparticles can be precipitated out of the sol using polycationic peptides such as Poly-L-Lysine (PLL) or (ii) the sol can undergo further condensation and polymerization reactions leading to continuous glass like gels which can be lyophilized to yield sol-gel based nanogels.
Figure 4. General Scheme for synthesis of sol-gel based nanoparticles
**Polycationic peptide protocol**

“The polycationic peptide protocol” is based on the use of polycationic peptides such as Polylysine for precipitating out sol-gel nanoparticles from a colloidal solution of TMOS (Figure 5). This protocol is a modified protocol based on methods used by Naik et al. (2004) [8].

The following standard procedure was used for synthesis of all nanogels using polypeptides:

TMOS (sigma, >99 % purity) was hydrolyzed by adding 1mM HCl (1:5 ratio) and sonicated for 30 minutes. Then 50 mM of Potassium Phosphate buffer pH 7 with the desired protein or molecule was mixed with the hydrolyzed TMOS solution. The mixture was left standing for 5 minutes at room temperature. 1ml deionized water was added to terminate the reaction. The solution was washed by centrifugation 3 times. After the final wash the particles were re-suspended in phosphate buffer. To make control nanogels no protein is added to the buffer.

When polylysine is used as the silica precipitating agent in the polycationic protocol then it would be referred to as “Polylysine protocol”.
Phosphate Buffer + Protein/Drug + Cationic peptide + TMOS solution.

Reaction time 5 min

add water → centrifuge → wash → Resuspended in buffer → Nanogels

Figure 5. Synthesis of nanogels using Polycationic peptide protocol.
Lyophilization Protocol

“The lyophilized protocol” involves encapsulating the desired molecules inside a polymeric sol-gel matrix followed by a stage where the matrix is dried and finally lyophilizing the dried gel matrix (Figure 6).

The following standard procedure was used for synthesis of all nanogels using lyophilization:

TMOS was hydrolyzed by adding 2mM HCl (2:1 ratio) and sonicated for 30 minutes.

Desired concentration of protein or small molecule was added to 50mM phosphate buffer pH 7. The buffer/protein mixture was added to hydrolyzed TMOS (1:1 ratio) and mixed and casted in desired container. The solution formed uniform gels in 30 seconds to 1 minute. The casted gel was washed with buffer 3 times to remove side products from surface of the gel. For control gels no protein was incorporated in the buffer.

To make nanogels, the sol-gel films were slightly crushed using a spatula and dried at room temperature for 12 hours and lyophilized for 5 hours (LABCONCO, freezone).
Figure 6. Synthesis of nanogels using Lyophilization protocol.
CHAPTER 3

PHYSICOCHEMICAL CHARACTERIZATION OF NANOGELS

The application of any nanocarrier platform requires high degree of control over their physicochemical properties. We performed systematic physicochemical studies including size distribution and aggregation, protein stability, protein/sol-gel interactions and protein release from sol-gel. The synthesis of particles was studied by varying different conditions like temperature changes, varying buffer and salt concentrations, different chain lengths of cationic polypeptides. Size characterization, particle morphology, size distribution and aggregation was studied by Transmission Electron Microscopy (TEM) combined with Dynamic Light Scattering (DLS). Protein stability, protein sol-gel interaction and release were studied using UV/Visible absorption spectroscopy. Characterization of surface properties of nanoparticulate carriers is complicated by charges of the different components of the nanospheres, the composition of the dispersion medium, and the extent of binding of biomolecule onto the nanoparticle. Thus we use Zeta potential measurements to study the surface properties and formulation stability of the particles.
EXPERIMENTAL PROCEDURES

Dynamic Light Scattering (DLS) and Zeta Potential Analysis

DLS measurements were performed on a 90 plus particle size analyzer (Brookhaven Instruments Corporation) employing a 90° scattering angle and a 35 mW incident laser (658 nm). Small amounts of nanogels (1-2mg) were suspended in deionized water and sonicated for 15 minutes before taking measurements. The reported diameter is the average of 5 runs for each sample.

Zeta Potential measurements were done by adding small amount (1-2mg) of nanogels in 1mM KCl solution and sonication for 15 minutes. (Brookhaven Instruments Inc. Zeta Plus).

Transmission Electron Microscope (TEM) Studies

Dilute samples used for DLS measurements were mixed in a 1:1 ratio with 1% uranyl acetate, sonicated for 10 minutes. Then 5ul was put on a carbon film coated copper grid, 300 mesh (electron microscopy sciences, Hatfield, PA) and the excess solution was removed immediately using absorbent paper. The grids were dried overnight and viewed under the transmission electron microscope (JEOL, model JEM-1200 EX). Images were taken with a SIA–L3C CCD camera (Scientific Instruments and Applications, Inc.) using the software Maxim DL5 (Diffraction Limited, Ottawa, Canada).
SIZE AND ZETA POTENTIAL ANALYSIS OF NANOGELS USING DYNAMIC LIGHT SCATTERING

DLS studies have indicated that different populations are present in nanogel solutions prepared using the Lyophilization method. The particle diameter ranges from 80 to 300nm.

For particles prepared using the polylysine protocol the particles range in size from 300 to 500nm with multimodal population.

These measurements correlate with TEM images (Figure 7 and 8) for both kinds of nanogels showing multiple populations.

Zeta potential (a measure of colloidal stability) measurements indicate that the lyophilized nanogels have a negative charge with zeta potential of -30mV, whereas PLL precipitated nanogels have a lower colloidal stability with a zeta potential of -8mV.

It was found that protein or the surfactant Polyethylene Glycol (PEG) (PEG is a steric stabililizer with various advantages discussed in Chapter 4) incorporation inside nanogels during the gelation step has no effect on zeta potential, indicating that incorporation of molecules inside the nanogel matrix does not affect its colloidal properties.
SIZE ANALYSIS OF NANOAGELS USING TRANSMISSION ELECTRON MICROSCOPE

Figure 7. TEM micrograph of control Nanogels synthesized using the Lyophilization protocol. The average nanoparticle size range is 50-100 nm, Scale Bar: 0.5 μm.

Figure 8. TEM micrograph of control nanogels using the Polylysine protocol. The average nanoparticle size range is 100-300nm, Scale Bar: 0.5 μm.
Studies from Transmission Electron Microscope (Figure 7 and 8) suggest that the lyophilized nanogels are uniformly dispersed circular particles with a size range of 10nm to 200nm most of the particles being 60nm. Presence or absence of encapsulated proteins or molecules inside the nanogels did not have a considerable effect on the nanogel’s size and distribution as shown in further chapters. The polylysine precipitated nanogels have a slightly larger size of 100 to 300 nm.

**Characterization of Nanogels under different conditions.**

The size dependence on Phosphate concentration in the range 3-200 mM was tested and there was no correlation with the resultant nanogel size (Table 1). Slight differences seen in the particle size are attributed to error in the instrument and changes due to solution stability.

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<th>Phosphate Buffer Concentration</th>
<th>Effective Diameter (nm)</th>
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<tr>
<td>3mM</td>
<td>143</td>
</tr>
<tr>
<td>10mM</td>
<td>138</td>
</tr>
<tr>
<td>30mM</td>
<td>207</td>
</tr>
<tr>
<td>100 mM</td>
<td>240</td>
</tr>
<tr>
<td>200 mM</td>
<td>210</td>
</tr>
</tbody>
</table>

*Table 1. Size of nanogels dispersed in phosphate buffers of different concentrations as obtained from Dynamic Light scattering instrument.*
Tests utilizing Potassium Chloride, Citrate buffer, TRIS- HCl as buffers for suspension of nanogels resulted in similar results and showed no effect on particle size of the particles.

The solubility of nanogels was also probed under different pH conditions (pH range: 5-7.5) and it was seen that there was no marked effect on the stability and size distribution of the nanogels when exposed to these pH conditions. Exposure to highly acidic conditions (pH 2) had no effect whereas exposure to highly alkaline conditions (pH 10-12), show smaller sizes of nanogels. This effect is discussed in more detail in later sections of this chapter.

As discussed earlier the nanogels synthesized from the Polyllysine protocol have a tendency to aggregate and are not colloidally stable for long periods of time (as compared to the nanogels produced from Lyophilization protocol). The stability and size of these particles were studied using the surfactant Polyethylene Glycol (PEG) to decrease aggregation and improve stability. Addition of increasing concentrations of PEG-400 decreased the size of the particles as tested by the DLS, showing low aggregation (Table 2). The Zeta Potential also became increasingly negative for the nanoparticles showing higher colloidal stability due to low aggregation (Table 3).

<table>
<thead>
<tr>
<th>[PEG-400] (%v/v)</th>
<th>Effective diameter of ngs (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>800</td>
</tr>
<tr>
<td>1.25</td>
<td>380</td>
</tr>
<tr>
<td>2.5</td>
<td>500</td>
</tr>
<tr>
<td>3.75</td>
<td>450</td>
</tr>
<tr>
<td>5</td>
<td>390</td>
</tr>
</tbody>
</table>

Table 2. The change in effective diameter of the nanoparticles with increasing amounts of PEG-400 added into suspending buffer. The effective diameter of the nanoparticles decreases as the PEG content increases.
Table 3. The change in zeta potential of nanogels with increasing amounts of PEG-400 added into the suspending buffer. The zeta potential of the nanoparticles becomes increasingly negative as higher amounts of PEG added suggesting greater colloidal stability.

<table>
<thead>
<tr>
<th>[PEG-400] (% v/v)</th>
<th>Zeta Potential of ngs (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-8</td>
</tr>
<tr>
<td>0.25</td>
<td>-16</td>
</tr>
<tr>
<td>0.5</td>
<td>-13</td>
</tr>
<tr>
<td>0.75</td>
<td>-19</td>
</tr>
<tr>
<td>1</td>
<td>-27</td>
</tr>
</tbody>
</table>

**RELEASE STUDIES ON ENCAPSULATED MOLECULES –Polylysine protocol**

Release of encapsulated molecules was tested using UV/Visible spectroscopy. The protein Met - Myoglobin (Met-Mb) was incorporated inside the nanogels synthesized using the polylysine protocol (encapsulation confirmed by UV spectra showing characteristic absorbance peak at 407 nm) (*Figure 9*) and the release of protein from the nanogels was tested by suspending the particles in pH 7 phosphate buffer and spinning down the particles at different time intervals to measure the Met-Mb characteristic O.D at 407nm in the supernatant (*Figure 10*).
Figure 9. UV/Visible spectra of Met-Mb in solution and Met-Mb encapsulated nanogels. Characteristic peak of Met-Mb at 407nm suggests the encapsulation of the protein inside nanogels.
About 40% of the protein is released within 5 minutes. The remaining is released through a slow and steady rate. The release of the protein from polylysine protocol nanogels is spontaneous.
Once synthesized the encapsulated protein readily diffuses out of the porous matrix. Encapsulation of other filling molecules such as surfactants (PEG, cellulose, other polymers) inside the matrix may be able to provide more control over release properties of these peptide precipitated nanogels for application where the molecules are required to stay inside the nanogels for a longer period of time.

**RELEASE STUDIES ON ENCAPSLATED MOLECULES (Sol-gel films)**

Protein release studies were carried out on sol-gel films to evaluate release properties of the sol-gel matrix and gain insight into similar properties of synthesized nanoparticles. The studies were done on Hemoglobin (Hb) (Hb is discussed in detail in Chapter 5) encapsulated nanogels using UV/Visible spectroscopy. These studies were performed under different pH conditions. The effect of concentration and temperature was also studied for possible changes in release patterns. Sol-gel films containing Hemoglobin were casted on sides of standard cuvettes using our standard sol-gel procedure and bathed in phosphate buffers with varying pH’s (pH 2-12), ionic concentrations (5mM, 50mM, 200mM, 500mM) and temperatures (10°C, 23°C, 35°C). The bathing buffer was separated at different time intervals and probed using UV/Visible spectra to test release of protein into the buffer detected by characteristic Hb absorbance at 405nm. The measured absorbance was correlated to % protein released over time.
Results

There is no evidence for Hb release from the sol-gel matrix under buffer conditions of pH 2-8, indicating it remains trapped within the matrix. However there is substantial release under alkaline conditions (Figure 11). About 60% of the protein is released from the sol-gel film in 2 hours when exposed to pH 12. (Table 4)

Table 4. Release of Hb from sol-gel films under different pH conditions. Release of Hb from sol-gel films was measured by taking O.D of bathing buffer at 405nm over different time intervals and correlated to % release.

<table>
<thead>
<tr>
<th>pH</th>
<th>Hemoglobin release</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td>NO RELEASE</td>
</tr>
<tr>
<td>6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.6 % released in 2 hours</td>
</tr>
<tr>
<td>12</td>
<td>Substantial release, about 0.5 % /min. Approx. 60% released in 2 hours.</td>
</tr>
</tbody>
</table>
Similar release experiments were performed with Myoglobin encapsulated sol-gel films and it was seen that there is no release of Myoglobin from sol-gel films at pH 7 but substantial release after exposure to pH 12.4 for 1.5 hours (Figure 12).

**Figure 11.** UV/Vis spectra for release of Hb at pH 7 and pH 12.4. Release of Hb from sol-gel films was determined by measuring the OD of bathing buffer at 405nm over different time intervals and correlated to % release.
Figure 12. UV/Vis spectra for release of Mb from sol-gel films. Release of Mb from sol-gel films was determined by measuring the OD of bathing buffer at 407nm over different time intervals.
Release profiles of proteins were tested at various conditions of temperatures and ionic strength of buffers, and long term storage. The concentration of bathing buffer (5mM-500mM) and temperature (4°C-60°C) had negligible effect on immediate release of Hb and Mb proteins. When exposed to very high temperatures for extended periods of time there was only about 0.5% release of Hb and about 2% release of Mb from the sol-gel films. Short term storage (1 day to 1 week) or Long Term Storage of sol-gels (1 week to 2 months) does not show any release of encapsulated proteins into the buffer.

The release of a small molecule, the potential anticancer drug Distamycin A (also discussed in Chapter 6) from sol-gel films was tested at pH 7 and pH 12 (Figure 13). Distamycin A was encapsulated inside sol-gel films using the standard protocol for making sol-gel films and release into bathing buffer was tested by UV/Vis spectroscopy to see characteristic peak at 303nm. It is seen that the release of small molecules is also higher at alkaline conditions.

![Figure 13. Release of small molecule Distamycin A from sol-gel films. Release of Distamycin A was determined by measuring O.D of bathing buffer at 303nm. The O.D was correlated to % release over time.](image-url)
The sol-gel matrix seems to degrade at alkaline pH conditions. This conclusion is supported by the observation that sol-gel films tend to thin down after exposure to basic buffer and is completely degraded/diminished after bathing in this buffer for 2-3 days. Moreover when nanogels were synthesized from these films and suspended in pH 12 buffer, the effective diameter measured using DLS decreased over time and the diameter was close to zero in a period of 2 hours (Table 5). Similar degradation of particles was noticed in nanogels produced from both Lyophilization and Polylysine protocols.

Table 5. Effective diameters of nanogels exposed to basic conditions over a function of time. The size of the nanogels decreases with increasing exposure time to pH 12 phosphate buffer.

<table>
<thead>
<tr>
<th>Time (min) of exposure of nanogels to pH 12 buffer</th>
<th>Effective Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>360</td>
</tr>
<tr>
<td>60</td>
<td>18</td>
</tr>
<tr>
<td>120</td>
<td>8</td>
</tr>
<tr>
<td>140</td>
<td>0</td>
</tr>
</tbody>
</table>

The effect of concentration (5mM-500mM) and temperature (10°C-35°C) was found to have negligible difference on release patterns in a period of 2 hours. Higher temperature of about 52°C does not show any release of encapsulated protein immediately, however continuous exposure for about 60 minutes to high temperature shows only 0.5% release of Hb and approximately 2% release of Mb from sol-gel films. A slightly higher % release of Mb is expected in comparison to Hb (over extended periods of time) because of the comparatively smaller size of Mb.
Conclusions

After analysis of the nanogels resulting from two different approaches (Polylysine and Lyophilization) it was concluded that the particle size of polylysine precipitated nanogels have a wider size distribution of 300-500nm whereas particles synthesized using the lyophilization protocol have a size ranging from 50nm -100nm. Nanogels synthesized from polylysine protocol have a tendency to aggregate rapidly as seen from DLS studies and TEM studies. Although macromolecules can be easily encapsulated inside these nanogels, they rapidly diffuse out of the seemingly porous matrix of these particles. In comparison nanogels synthesized using Lyophilization protocol are much more stable with regard to aggregation and shows greater colloidal stability (indicated by the zeta potential of -30mV) as well as better encapsulation (entrapment inside the matrix) of macromolecules. Proteins such as Hb do not diffuse out of the sol-gel matrix under most working/biologically relevant conditions. In spite of being confined to the sol-gel matrix the protein remains active and functional (as shown from studies described in later chapters). Moreover the degradation of the sol-gel matrix at alkaline conditions can be further evaluated for potential therapeutic advantage for delivery of encapsulated molecules for various applications.
Synthesis of nanogels using different precipitating agents

Synthesis of silica nanoparticles utilizing cationic precipitating peptides such as R5 isolated from Diatoms have been studied earlier [6,8] and showed potential for development for various applications. We explored this biomimetic approach, using the polycationic silica precipitating agent Polylysine and then expanded it to other cationic agents such as Lysine, Arginine and Histidine. All the nanoparticles were synthesized using the standard polycationic peptide protocol using equivalent concentrations of precipitating peptides (10mg/ml). The resultant nanogels size was determined using DLS measurements (Brookhaven Instruments Corporation BIC) and TEM micrographs (JEOL, model JEM-1200 EX).

Results

The resulting sol-gel based nanoparticles using Lysine gave a smaller average size of 100-300 nm as compared to a larger size of 300-500 nm when using PLL based nanogels. Particles produced using Arginine have similar size distribution to that of Lysine (100-300 nm), yet display a lower colloidal stability and low uniformity with high aggregation tendencies. The use of Histidine for silica precipitation did not provide any defined shapes and showed high aggregation tendencies (Table 6 and Figure 14).
Table 6. Differences between nanogels synthesized from different precipitating agents

<table>
<thead>
<tr>
<th>Precipitating agent</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polylysine</td>
<td>Circular uniformly dispersed nanogels. Size: 300-500 nm.</td>
</tr>
<tr>
<td>Arginine</td>
<td>Not as uniformly circular, smaller in size as compared to Polylysine. Size: 100-300 nm. Aggregates readily. Low colloidal stability.</td>
</tr>
<tr>
<td>Histidine</td>
<td>No defined shapes formed. High aggregation.</td>
</tr>
</tbody>
</table>
Figure 14. a. Nanoparticles made by polylysine Average Size: 400nm, Scale Bar: 500 nm; b. Nanoparticles made by Lysine, Average Size: 100nm, Scale Bar: 2µm; c. Nanoparticles made by Arginine, Average Size: 100nm -300 nm, Scale Bar: 500 nm.
Conclusions

Precipitation of nanogels using cationic peptides other than polylysine resulted in smaller sizes (arginine and lysine), although over long periods of time they show low colloidal stability. Monomeric cationic amino acids are able to produce particles and offer an economic advantage over longer chain polypeptides. Use of arginine and poly-arginine as precipitating agents is of much interest as this approach may offer a potential therapeutic advantage due to polyarginine’s well-known cell penetrating properties for drug delivery [11-14].
CHAPTER 4

SURFACE MODIFICATION AND PEG DECORATION OF NANOGE LS

In various studies it has been sometimes found that, after synthesizing nanoparticles, aggregation among particles took place when they were re-suspended in an aqueous solution, because the suspended silica particles formed agglomerates, probably due to (interparticle) hydrogen bonding [15]. Steric hindrances, by the use of polymers (steric stabilizers) adsorbed or grafted onto particle surfaces, have been one answer to the problems posed by aggregations among particles in an aqueous suspension. In this case, direct contacts among particles are prevented by the polymer chains extending into the medium. Therefore, aggregation of particles will not be formed, or the rate of coagulation will be decreased, depending on the degree of coverage of polymers on the particles and the thickness of the polymer layer [16-18]. We have used polyethylene glycol (PEG) to coat our nanogels (Figure 15). PEG acts as a steric stabilizer, its polymeric polyethylene chains extend far into the surrounding medium. Thus, in an aqueous suspension, the aggregation among the nanoparticles is prevented by the repulsion force and solvation layer of the PEG surface moiety [19]. Some other important advantages of having PEG coatings on the silica nanoparticles are due to their applications inside biological samples. PEG is nontoxic, and its attachment to silica nanoparticles provides a biocompatible and protective surface [20]. Also, the PEG coatings reduce protein and cell adsorption onto the particles and can reduce the rate of clearance of PEG coated materials, hence increasing the particle circulation time in bodily fluids for in vivo applications [21].
PROCEDURE

Synthesis of PEG coated nanogels

TMOS was hydrolyzed by adding 2mM HCl (2:1 ratio) and sonicated for 30 minutes. Desired concentration of PEG 400 was added to 50mM phosphate buffer pH 7. The buffer/PEG mixture was added to the hydrolyzed TMOS (1:1 ratio) and mixed. The solution formed uniform gels in 30 seconds to 1 minute. The casted gel was washed with buffer 3 times.

To make nanogels, the sol-gel films were slightly crushed using a spatula and dried at room temperature for 12 hours and lyophilized for 5 hours (LABCONCO, freezone).

Dynamic Light Scattering and Zeta Potential Analysis

DLS measurements were performed on a 90 plus particle size analyzer (Brookhaven Instruments Corporation) employing a 90° scattering angle and a 35 mW incident laser (658 nm). Small amounts of nanogels (1-2mg) were suspended in deionized water and sonicated for 15 minutes before taking measurements. The reported diameter is the average of 5 runs for each sample. Zeta Potential measurements were done by adding small amount (1-2mg) of nanogels in 1mM KCl solution and sonication for 15 minutes. (Brookhaven Instruments Inc. Zeta Plus).
Transmission Electron Microscope Studies

Dilute samples used for DLS measurements were mixed in a 1:1 ratio with 1% uranyl acetate, sonicated for 10 minutes. Then 5ul was put on a carbon film coated copper grid, 300 mesh (electron microscopy sciences, Hatfield, PA) and the excess solution was removed immediately using absorbent paper. The grids were dried overnight and viewed under the transmission electron microscope (JEOL, model JEM-1200 EX). Images were taken with a SIA–L3C CCD camera (Scientific Instruments and Applications, Inc.) using the software Maxim DL5 (Diffraction Limited, Ottawa, Canada).

Fourier Transform Infrared Spectroscopy (FTIR) Studies

Evidence of the PEG coating was given by FTIR spectra of the dried nanogel particles, recorded by a FTIR-8400S Shimadzu. The samples were prepared by mixing the pure PEG polymers or nanogels with KBr by grinding, and then placed some of the powder mixture into a metal mount to form a pellet in the mount by applying pressure to both ends. The prepared mount was then placed into the FTIR system, for analysis.

Rhodamine-PEG binding tests using UV Visible Spectroscopy

Control nanogels were prepared using standard Lyophilization protocol (given in Chapter 2). Synthesized control nanogels were incubated in a solution of Rhodamine-PEG 5000 (Nanocs Inc.). The nanogels were spun down using a centrifuge at periodic intervals and UV Visible spectra of the supernatant was taken using Shimadzu UV-2450.
Results - PEG incorporation within nanogels.

Figure 16. FTIR spectra of a. pure sol-gel nanoparticles, b. pure PEG 400, c. sol-gel nanoparticles modified with 1% v/v PEG 400, d. sol-gel nanoparticles modified with 2.5% PEG 400, e. sol-gel nanoparticles with 5% PEG 400, f. sol-gel nanoparticles with 10% PEG 400. The spectrum of PEGylated sol-gel nanoparticles shows a new absorption peak at 2882 cm\(^{-1}\), corresponding to one of the characteristic peaks of PEG 400. These peaks suggest the binding of PEG to the silica particles.

Pure silica particles possess characteristic peaks at 796, 950, 1065, 1400, 1633, and 3400 cm\(^{-1}\).

Most of the peaks associated with PEG are obscured by overlapping peaks of the pure silica
nanoparticles. However the spectrum of PEGylated sol-gel nanoparticles (Fig. 16(c-f)) shows a new absorption peak at 2882 cm\(^{-1}\), corresponding to one of the characteristic peaks of PEG 400. These peaks suggest the binding of PEG to the silica particles.

TEM micrographs showed that the PEG encapsulated nanoparticles are more stable over time. After storage for 2 days without disturbance the particles with PEG were more uniformly distributed and disaggregated as compared to nanogels without PEG (Figure 17).

**Figure 17.** TEM micrographs of **a.** aggregated nanogels (no PEG) which were stored for 2 days, Scale Bar: 1µm; **b.** nanogels with PEG incorporated inside which were stored for 2 days, Scale Bar: 1µm.
**PEG Binding Tests**

The binding of PEG to the surface of nanogels was investigated by incubating control nanogels with a solution of Rhodamine-PEG 5000 adduct (Nanocs inc). (Illustration shown below). Rhodamine-PEG 5000 adduct solution was incubated with nanoparticles for 2 hours and the solution was centrifuged away from the nanogel particles and the O.D of the supernatant (at characteristic wavelength of 558 nm) taken at different time intervals was measured (Figure 18).

![Diagram](image)

**Figure 18.** Rhodamine-PEG 5000 adduct solution was incubated with nanoparticles for 2 hours and the solution was centrifuged away from the nanogel particles. Pictures of a. nanogel with Rhodamine PEG 5000 adduct mixed in the buffer; b. spun down nanogels incubated with Rhodamine PEG 5000 adduct for 2 hours.
As seen in Figure 19, the decrease in O.D at 558nm shows that approx. 50% of the Rhodamine-PEG adduct was bound to the surface of the particles after 2 hours. After continuous washing the PEG Rhodamine adduct is still bound to the surface of nanogels indicating that PEG gets adsorbed to the surface of the nanogels.

**PEG binding tests using Sol-Gel Blocks**

Sol-gel blocks were made by mixing hydrolyzed TMOS (with 2mM HCl) and 50mM pH 7 phosphate buffer using standard protocol. The gels were molded in glass vials, washed 3 times with buffer and allowed to stand for 12 hours to age. Equivalent concentrations of Rhodamine B and Rhodamine-PEG adducts were added to the top surface of two separate sol-gel blocks and diffusion of added solutions into the gel was observed periodically over a period of 2 hours.
Figure 20. **a.** pure sol-gel with buffer; **b.** sol-gel with Rhodamine dye and Rhodamine PEG-5000 mixed in the buffer; **c.** sol-gel after 2 hours **d.** sol-gel after 2 hours inverted view; **e.** sol-gel after 2 hours slanted view. After 2 hours of incubation of sol-gel blocks I and II with Rhodamine dye and Rhodamine–PEG adduct respectively, Rhodamine dye almost completely diffuses inside the sol-gel whereas the Rhodamine-PEG adduct stays mostly at the surface of the sol-gel.
As can be seen in Figure 20, after 2 hours of incubation of sol-gel blocks I and II with Rhodamine dye and Rhodamine–PEG adduct respectively, Rhodamine dye almost completely diffuses inside the sol-gel whereas the Rhodamine-PEG adduct stays mostly at the surface of the sol-gel. The Rhodamine PEG adduct is also not seen in the buffer on top of the sol-gel, as compared to the Rhodamine dye which is evenly distributed throughout the sol-gel and the buffer on top of it. This indicates that PEG binds to the surface of sol-gel and does not diffuse inside.
SURFACE MODIFICATION OF NANOGELS USING PHOSPHONATE AND AMINE GROUPS.

Surface modification of nanogels was done to reduce aggregation of particles and to also provide linking groups on the surface of the nanogels for attachment of different therapeutic ligands. When amine and phosphonate groups are present on the surface of particles there is an elimination of back bonding due to interactions between the amine and phosphonate groups on the particle surface, thus increasing the shear plane and electrostatic repulsion of the particles. Amine groups can be attached using 3-Amino propyl triethoxy silane (APTES) and phosphonate groups by using 3 -(trihydroxysilyl) propyl methyl phosphonate (THPMP) (Figure 21) [22].

![Diagram of surface modification using APTES and THPMP](image)

**Figure 21.** a. Attachment of APTES; b. Attachment of APTES and THPMP to nanogels. When amine and phosphonate groups are present on the surface of particles there is an elimination of back bonding due to interactions between the amine and phosphonate groups on the particle surface, thus increasing the shear plane and electrostatic repulsion of the particles [Bagwe et al., 2006].
**Procedure**

A mixture of APTES, THPMP and TMOS in ethanol was sonicated for 20 minutes. The resulting hydrolyzed TMOS solution containing the different organosilane groups was mixed with 50mM pH 7 phosphate buffer in a 1:1 ratio. This solution gelled within 1 minute. The resulting sol-gel was dried for 24 hours and lyophilized for 5 hours resulting in modified nanogels (**Figure 22**). The modified nanogels were tested for size (DLS) and zeta potential to evaluate their colloidal stability.

![Figure 22. Synthesis of APTES and THPMP modified nanogels.](image-url)
Results

The size of particles as seen from the DLS measurements (Figure 23) increased with increasing concentrations of amine groups (APTES) and decreased with increasing concentrations of phosphonate groups (THPMP).

**Figure 23.** Effect of increasing amine and phosphonate modification on particle diameter of nanogels. The size of particles as seen from the DLS measurements increased with increasing concentrations of amine groups (APTES) and decreased with increasing concentrations of phosphonate groups (THPMP).
The zeta potential of the particles increases and becomes less negative with increasing amine concentration (APTES) showing less colloidal stability. On the other hand the zeta potential becomes increasingly negative with increase in phosphonate concentration (THPMP) suggesting higher colloidal stability (Figure 24).

**Figure 24.** Effect of increasing amine and phosphonate modification on zeta potential of nanogels. The zeta potential of the particles increases and becomes less negative with increasing amine concentration (APTES) showing less colloidal stability. Zeta potential becomes increasingly negative with increase in phosphonate concentration (THPMP) suggesting higher colloidal stability.
Conclusions

Our studies on surface modification of nanogels using linkers like PEG, APTES and THPMP suggest that the surface of particles can be modified to improve the properties of these nanogels such as aggregation, and linkage to other groups to improve targeting. Furthermore these modifications can be performed using mild approaches (room temperature and physiological pH) so as not to affect the nature of the encapsulated molecules.
CHAPTER 5
SOL-GEL AND NANOGEL ENCAPSULATION OF MACROMOLECULES

Biological agents can be incorporated inside the sol-gel network and nanogels due to its unique favorable properties towards proteins and enzymes. Here we have incorporated different macromolecules inside the sol-gel and nanogel matrices and tested if the proteins are intact and functional.

**Incorporation of Hemoglobin inside sol-gels and nanogels.**

Hemoglobin (Hb) is an iron-containing oxygen-transport metalloprotein in the red blood cells of most vertebrates as well as the tissues of some invertebrates. Hemoglobin in the blood carries oxygen from the respiratory organs (lungs or gills) to the rest of the body (i.e. the tissues) where it releases the oxygen for providing energy to power the functions of the organism in the process called metabolism [23,24].

In most vertebrates, the hemoglobin molecule is an assembly of four globular protein subunits. Each subunit is composed of a protein chain tightly associated with a non-protein heme group (Figure 25). A heme group consists of an iron (Fe) ion held in a heterocyclic ring, known as a porphyrin.

In adult humans, the most common hemoglobin type is a tetramer (which contains 4 subunit proteins) called hemoglobin A (Figure 25), consisting of two α and two β subunits non-covalently bound, each made of 141 and 146 amino acid residues, respectively. This is denoted as α2β2. The subunits are structurally similar and about the same size. Each subunit has a molecular weight of about 16,000 Daltons, for a total molecular weight of the tetramer of about
64,000 Daltons. Hemoglobin A is the most intensively studied of the hemoglobin molecules [24-26].

Figure 25. a. Structure of human hemoglobin. The proteins’ α and β subunits are in red and blue, and the iron-containing heme groups in green; b. Structure of Heme b group.

The iron ion may be either in the Fe$^{2+}$ or in the Fe$^{3+}$ state, but ferrihemoglobin (methemoglobin) (Fe$^{3+}$) cannot bind oxygen. While binding, oxygen temporarily and reversibly oxidizes (Fe$^{2+}$) to (Fe$^{3+}$), oxygen temporarily turns into superoxide, thus iron must exist in the +2 oxidation state to bind oxygen. If superoxide ion associated to Fe$^{3+}$ is protonated, the hemoglobin iron will remain oxidized and incapable of binding oxygen. In such cases, the enzyme methemoglobin reductase will be able to eventually reactivate methemoglobin by reducing the iron center.

It is known that methemoglobin (iron is in 3+, ferric state) absorbs at 405nm. In this state the hemoglobin cannot bind oxygen. Deoxyhemoglobin (+2 ferrous state) absorbs at 430 nm, this is not bound to oxygen but it can when exposed to oxygen. Oxyhemoglobin (+2 ferrous state) absorbs at 415nm. This is when Hb is bound to oxygen. Hemoglobin can shift between different ligation and oxidation states depending on the environment it is exposed to and this property is vital for it to perform its function in the body [25-27].
In this study Met-Hemoglobin was incorporated inside nanogels and sol-gel films using the standard protocol for nanogels and sol-gel synthesis. To these nanogels and sol-gel films and nanogels, sodium dithionite (DTT) (a reducing agent) was added to change the oxidation state and further the nanogels were exposed to oxygen to change the ligation state.

The position of the absorbance peak due to the HEME changes under different ligation states and oxidation states showing that the protein is functional inside the nanogels and the sol-gel film (Figure 26). It is seen that encapsulated Met-Hb starts out with an absorbance peak at 405nm, which shifts to 430 nm after addition of DTT (which changes iron from +3 to +2 oxidation state). Upon exposure to oxygen the peak returns to 415nm indicating the binding of oxygen and conversion to oxy-hemoglobin.

These results also show that the diffusion into the sol-gel is very efficient and that water and added small molecules (such as DTT) can reach the encapsulated protein.
Figure 26. UV/Vis spectra of Hemoglobin in solution, encapsulated inside sol-gel films, and nanogels. It is seen that encapsulated Met-Hb starts out with an absorbance peak at 405 nm, which shifts to 430 nm after addition of dithionite (which changes iron from +3 to +2 oxidation state). Upon exposure to oxygen the peak returns to 415 nm indicating the binding of oxygen and conversion to oxy-hemoglobin. The position of the absorbance peak due to the HEME changes under different ligation states and oxidation states showing that the protein is functional inside the nanogels and the sol-gel film.
Incorporation of Glucose Oxidase

The glucose oxidase enzyme (GOx) is an oxido-reductase that catalyzes the oxidation of glucose to hydrogen peroxide (H₂O₂) and D-glucono-δ-lactone [28,29].

\[
\beta\text{-d-glucose} + O_2 \xrightarrow{\text{Glucose Oxidase}} \text{hydrogen peroxide} + \text{d-glucono-δ-lactone}
\]

In cells, it helps in breaking sugar down into its metabolites. Glucose oxidase (often extracted from *Aspergillus niger*) is widely used for the determination of free glucose in body fluids (diagnostics), in vegetal raw material, and in the food industry. It also has many applications in biotechnology, typically enzyme assays for biochemistry including biosensors in nanotechnology [28-31].

GOx was incorporated inside the nanogels using our standard protocol for synthesis of nanogels (Figure 27).

![Synthesis of GOx encapsulated nanogels](image_url)

**Figure 27.** Synthesis of GOx encapsulated nanogels
The presence of GOx inside the sol-gel film was confirmed by checking characteristic protein UV/Vis peak at 280nm (Figure 28).

![Figure 28. UV/Vis spectra of Glucose Oxidase nanogels. The spectra shows characteristic protein peak at 280 nm suggesting that the GOx was encapsulated inside the nanogels.](image)

The activity of GOx encapsulated inside the synthesized nanogels was tested by exposing the nanogels to glucose and using an iodide assay for determination of H₂O₂ (measured spectroscopically at 352nm).

The sample containing hydrogen peroxide was mixed with Reagent A (6.5 g KI and 0.5 g NaOH and 0.05 g ammonium molybdate in 250 mL water, final pH 11) and Reagent B (5 g biphthalate in 250 mL water, the pH should be 4) in a 1:5:5 ratio. The mixture was allowed to develop color for 5 minutes before taking UV/Visible Spectra.
Our studies have showed that GOx when encapsulated inside the sol-gel matrix is functionally active and is able to generate H$_2$O$_2$ when it is exposed to 0.3 M glucose (Figure 29).

![Graph showing H$_2$O$_2$ concentration over time with a linear trend line y = 0.0235x.](image)

**Figure 29.** Continuous release of H$_2$O$_2$ from GOx encapsulated nanogels when exposed to 0.3 M Glucose. The nanogels were exposed to bathing buffer containing glucose. The concentration of H$_2$O$_2$ was determined by using an iodide assay for determination of H$_2$O$_2$ (measured spectroscopically at 352 nm). The rate of formation of H$_2$O$_2$ is 23.5 µM/min.

When the release rates of nanogels and sol-gel films were compared to that in solution with equivalent final concentrations of GOx (Figure 30), it was seen that GOx nanogels had the lowest rate with 18.6 µM/min and the sol-gel films had a slightly higher H$_2$O$_2$ release rate of 23.1 µM/min. The rate in solution is highest (37.9 uM/min) when compared to both nanogels and sol-gels. The observation may be attributed to the fact that it takes more time for the dextrose to diffuse into the sol-gel and nanogels and react with the entrapped enzyme and there is also a delay in the release of H$_2$O$_2$ produced as a result of the reaction.
Both the sol-gel and nanogels were actively producing $\text{H}_2\text{O}_2$ after a week of storage.

These results indicate that the sol-gel and nanogel network is capable of holding the protein intact inside without adversely affecting its biological function.

**Sol-gel Incorporation of Transferrin**

Transferrins are iron-binding blood plasma glycoproteins that control the level of free iron in biological fluids. Transferrin glycoproteins bind iron very tightly, but reversibly. Transferrin has a molecular weight of around 80 KDa and contains two specific high-affinity Fe(III) binding sites [32]. The affinity of transferrin for Fe(III) is extremely high at pH 7.4 but decreases progressively with decreasing pH below neutrality. When not bound to iron, it is known as "apotransferrin" [33].

Transferrin has various roles in diseases and is therefore of great interest to scientists for therapeutic purposes. An increased plasma transferrin level is often seen in patients suffering
from iron deficiency anemia. A decreased plasma transferrin can occur in iron overload diseases and protein malnutrition. An absence of transferrin results from a rare genetic disorder known as atransferrinemia; a condition characterized by anemia and hemosiderosis in the heart and liver that leads to many complications, including heart failure [34-36]. Transferrin is also associated with the innate immune system. It is found in the mucosa and binds iron, thus creating an environment low in free iron that impedes bacterial survival in a process called iron withholding. The level of transferrin decreases in inflammation [37]. Most recently, transferrin and its receptor have been shown to diminish tumour cells by using the receptor to attract antibodies [35, 38-40].

We have incorporated Transferrin inside sol-gel films and tested its function and stability utilizing a Phenanthroline assay for iron determination. 1, 10-Phenanthroline reacts with Fe\(^{2+}\) to form a characteristic orange-colored complex (Figure 31). The intensity of color development is directly proportional to the amount of Fe\(^{2+}\) in the sample which can be measured at 510 nm.

\[
3 \text{phen} + \text{Fe}^{2+} \rightarrow \text{Fe(phen)}_3^{2+}
\]

**Figure 31.** Chemical reaction of 1, 10-Phenanthroline used for iron determination. Ferrous ions react with 1, 10 Phenanthroline to form an orange colored complex with a characteristic OD at 510 nm.
Procedure

Transferrin encapsulated sol-gel films were prepared using the standard protocol for sol-gel films. The films were exposed to 50 mM pH 3 citric acid-phosphate buffer for 1 day. The bathing buffer was separated and mixed with 10% v/v Hydroxylamine HCl and 0.3% w/v 1,10 Phenanthroline in 5:1:1 ratio. Hydroxylamine serves to reduce the ferric ion to ferrous ion which can then react with 1,10 Phenanthroline and form the colored complex. The solution was allowed to mix for 5 minutes and the O.D at 510 nm was measured using a diode array UV Visible spectrophotometer (Agilent) (Figure 32).

![Figure 32. Phenanthroline assay for iron release test. The sol-gel film encapsulating the apotransferrin was bathed with 50mM pH 3 buffer. The bathing buffer was separated and mixed with 10% v/v Hydroxylamine HCl and 0.3% w/v 1, 10 Phenanthroline in 5:1:1 ratio. Hydroxylamine serves to reduce the ferric ion to ferrous ion which can then react with 1,10 Phenanthroline and form the colored complex. The solution was allowed to mix for 5 minutes and the OD at 510 nm was measured using a diode array UV-Visible spectrophotometer.](image)

The results showed that the iron held by the transferrin encapsulated inside the sol-gel is released after exposure to low pH indicating that the transferrin is functionally active even after entrapment inside the sol-gel matrix (Figure 33).
Figure 33. The release of iron from transferrin encapsulated inside sol-gel film when exposed to low pH, tested by Phenanthroline assay using spectroscopic measurement at 510nm.

A similar experiment was done with nanogels incorporated with transferrin. In a period of 2 hours of exposure to low pH almost all the iron was released from the transferrin entrapped inside the nanogels (Figure 34).
Figure 34. The release of iron from transferrin encapsulated inside nanogels when exposed to low pH, tested by Phenanthroline assay using spectroscopic measurement at 510 nm.

Compatibility of transferrin with the sol-gel matrix is promising because transferrin can be used in drug delivery as a conjugation linker to stabilize and improve the pharmacokinetics of certain therapeutically-important biomolecules. It efficiently transports these biomolecules and links with key cell receptors, providing intracellular delivery. Use of Transferrin with biodegradable nanoparticles such as ours gives rise to possibilities for various therapeutic applications exploiting the unique advantages of nanogels.
Conclusions

Fabrication of nanogels with macromolecules encapsulated within was achieved. There was no significant difference of nanogel properties with or without the encapsulated proteins. In spite of being confined to the sol-gel matrix, incorporated proteins are active and functional. Their small molecule substrate and products however can diffuse in and out of the sol-gel matrix. These results suggest that nanogels are an optimal choice for a macromolecule carrier matrix.
CHAPTER 6
SOL-GEL AND NANOGEL ENCAPSULATION OF SMALL MOLECULES

Application of various nanoparticles for the delivery of small therapeutic agents is still being explored by scientists and a lot of work needs to be done in the characterization and safety of nanoparticles being studied. Nanogels are suitable carriers for delivery of small molecules due to their properties. We have synthesized small molecule encapsulated nanogels by utilizing a very simple technique of trapping the drugs inside the nanogel network while the particles are being synthesized. The solgel network is able to encapsulate various kinds of drugs such as Ampicillin, Kanamycin, Thymoquinone and Distamycin A (Figure 35). Our release studies have shown that drugs are diffused out of the nanogel pores readily because of their small size.

![Figure 35](image)

Figure 35. Structure of a. Ampicillin, b. Kanamycin, c. Thymoquinone, d. Distamycin A.
Bacterial cell survival studies were performed using the synthesized antibiotic encapsulated nanogels using the following procedure.

**Procedure for Bacterial Cell survival studies**

*E. coli* was grown aerobically at 37°C for 12-16 h with shaking (250 rpm) in Luria-Bertani medium (LB). The saturated cultures were diluted in fresh LB medium starting at (OD 600) 0.05 (optical density) and incubated at 37 °C in Erlenmeyer flasks with vigorous shaking at 250 rpm as well until they reached a concentration of 2x 10^5 cells/ml. Each *E.coli* solution was incubated with different nanogels for a total time of 90 minutes. Four dilutions were made at each time interval and three 20 ul drops were plated from each dilution on solid agar plates. Plates were incubated overnight at 37° C and colonies counted. All results were compared to the control without any nanogels (Figure 36).

**Figure 36.** Illustration for bacterial cell survival studies of nanogels. An original inoculum of bacteria is incubated with samples being tested. For each time point the concentration of cells is determined by taking a sample of known volume from the original inoculum and preparing serial dilutions and plating for kinetic study. Number of bacteria at a given time point is calculated by multiplying no. of surviving colonies on plates by the reciprocal of dilution of samples.

\[
\text{Number of colonies (n) plate } \times \text{ reciprocal of dilution of sample (1/D)} = \text{number of bacteria /ml in the reaction mixture for a given time point.}
\]
Synthesis of Kanamycin Encapsulated nanogels and testing on E.coli.

Kanamycin is an aminoglycoside bactericidal antibiotic isolated from the bacterium Streptomyces kanamyceticus, and is used to treat a wide variety of infections [41].

Kanamycin interacts with the 30S subunit of prokaryotic ribosomes. It induces substantial amounts of mistranslation and indirectly inhibits translocation during protein synthesis [42, 43].

We have incorporated Kanamycin inside nanogels and tested their effect on E.coli bacterial cells. The following method was used to prepare Kanamycin encapsulated nanogels:

TMOS (sigma > 99%) was hydrolyzed using 2mM HCl for 30 minutes (2TMOS: 1HCl). Kanamycin sulfate (Gibco) was mixed in potassium phosphate buffer pH 7, 50mM. This solution was added to the hydrolyzed TMOS solution (1:1 ratio). This resulted in sol-gel blocks and Kanamycin was incorporated inside the sol-gel matrix during gelation. The sol-gels produced were dried at room temperature for 12 hours, slightly crushed using a spatula and lyophilized for 5 hours. (LABCONCO Free Zone 2.5).

Equivalent amounts of control and kanamycin nanogels were taken to test in bacteria. Final concentration of Kanamycin in the samples tested was 50ug/ml.

As seen in Figure 37, Control nanogels do not have adverse effects on the growth of bacteria, and Kanamycin encapsulated nanogels are able to deliver the antibiotic to the bacterial cells and demonstrate rapid killing.
Synthesis of Thymoquinone (TQ) encapsulated nanogels and testing on \textit{E.Coli}

Thymoquinone is a phytochemical compound found in the plant \textit{Nigella sativa}. It is known to exhibit antioxidant, anti-inflammatory and anti-cancer effects and has been shown to protect against heart, liver and kidney damage in animal studies [44-46]. It has been shown to suppress the proliferation of various tumor cells, including colorectal carcinoma, breast adenocarcinoma, osteosarcoma, ovarian carcinoma, myeloblastic leukemia, and pancreatic carcinoma [47–52], while being minimally toxic to normal cells [53].

Most drugs, especially those with hydrophobic nature such as Thymoquinone, when examined in human clinical trials fail because of either lack of safety or due to poor efficacy which could be in part due to poor bioavailability. Although a lot of natural products have served as leads for the

\textbf{Figure 37.} Effect of Kanamycin encapsulated nanogels on \textit{E.coli} cell survival. Control nanogels do not have adverse effects on the growth of bacteria, and Kanamycin encapsulated nanogels are able to deliver the antibiotic to the bacterial cells and demonstrate rapid killing.
majority of drugs, poor oral bioavailability has hindered their development without a suitable carrier [54].

Encapsulation of such a molecule would enhance cytotoxicity based on developments in drug delivery and nanotechnology.

We describe the design of biodegradable TQ encapsulated nanogels and characterize its effects on bacterial cell survival. We found that our sol-gel based technique and nontoxic synthesis method is compatible with a hydrophobic drug such as TQ, moreover TQ encapsulated nanogels are able to show antibacterial effect on *E.coli* cells. Successful results from these initial experiments opens ways for development of other hydrophobic plant based drugs using this technique.

**Procedure**

TMOS (sigma > 99%) was hydrolyzed using 2mM HCl for 40 minutes, 2TMOS : 1HCl. Thymoquinone (Sigma Aldrich) was mixed in potassium phosphate buffer pH 7 50mM. This solution was added to the hydrolyzed TMOS solution (1:1 ratio) resulting in a sol-gel blocks. Thymoquinone was incorporated inside the sol-gel matrix during gelation. The sol-gels produced were dried at room temperature for 12 hours, slightly crushed using a spatula and lyophilized for 5 hours (LABCONCO Free Zone 2.5).

The encapsulation of Thymoquinone was tested by measuring the UV/Vis absorbance of synthesized nanoparticles at characteristic wavelength of 258nm (Figure 38). Characteristic peak at 258nm confirmed the encapsulation of Thymoquinone inside the nanogels. The encapsulation is not 100% efficient, possibly due to the hydrophobic nature of the encapsulated drug, a portion of the drug may have released during washings of the nanogels.
Figure 38. UV/Vis spectra of Thymoquinone encapsulated nanogels. Characteristic peak at 258 nm confirmed the encapsulation of Thymoquinone inside the nanogels.
Equivalent amounts of control and Thymoquinone nanogels were taken to test in bacteria. Final conc. of Thymoquinone was 1mg/ml.

As can be seen in Figure 39, the Thymoquinone encapsulated nanogels are able to show killing towards *E.coli*, and the control nanogels do not affect the growth of bacteria showing that the carrier itself is non-toxic to the microorganism.

**Figure 39.** Effect of Thymoquinone encapsulated nanogels on bacterial cell survival. Thymoquinone encapsulated nanogels are able to show killing towards *E.coli*, and the control nanogels do not affect the growth of bacteria.
Synthesis of Ampicillin encapsulated nanogels and testing on *E.coli*.

Ampicillin is an antibiotic useful for the treatment of a number of bacterial infections. It is a beta-lactam antibiotic that is part of the aminopenicillin family. Ampicillin has been used extensively to treat bacterial infections since 1961. Until the introduction of ampicillin by the British company Beecham, penicillin therapies had only been effective against Gram-positive organisms such as *staphylococci* and *streptococci*. Ampicillin also demonstrated activity against Gram-negative organisms such as *H. influenzae*, *coli*forms and *Proteus* spp. [55,56].

Ampicillin is able to penetrate Gram-positive and some Gram-negative bacteria. It differs from penicillin G, or benzylpenicillin, only by the presence of an amino group. That amino group helps the drug penetrate the outer membrane of Gram-negative bacteria [57].

Ampicillin acts as an irreversible inhibitor of the enzyme transpeptidase, which is needed by bacteria to make their cell walls. It inhibits the third and final stage of bacterial cell wall synthesis in binary fission, which ultimately leads to cell lysis [57-59].

Since the antibacterial properties of Ampicillin are very well known we have used the antibiotic to test the effect of our nanogels using *E.coli* bacterial cells.

Ampicillin encapsulated nanogels were synthesized using the following procedure:

TMOS (sigma > 99%) was hydrolyzed using 2mM HCl for 40 minutes, 2TMOS: 1HCl. Ampicillin (Gibco) was mixed in potassium phosphate buffer pH 7 50mM. This solution was added to the hydrolyzed TMOS solution (1:1 ratio) resulting in a sol-gel blocks. Ampicillin was incorporated inside the sol-gel matrix during gelation. The sol-gels produced were dried at room
temperature for 12 hours, slightly crushed using a spatula and lyophilized for 5 hours. (LABCONCO Free Zone 2.5).

Equivalent amounts of control and ampicillin nanogels were taken to test in bacteria. Final conc. of ampicillin was 100ug/ml.

The bacterial studies (Figure 40) demonstrated the cytotoxicity of the ampicillin encapsulated towards E.coli cells.

![Figure 40](image)

**Figure 40.** Effect of Ampicillin encapsulated nanogels on E.coli cell survival. Ampicillin encapsulated nanogels are able to show killing towards E.coli, and the control nanogels do not affect the growth of bacteria.

**Incorporation of Distamycin A in Sol-gel and Nanogels**

Distamycin A is an antibiotic produced by *Streptomyces distallicus* the structure of which was identified and confirmed by total synthesis by Arcamone et al. (1964). Partially purified
preparations of Distamycin A exerted an inhibitory action on the development of experimental tumors. The purified crystalline antibiotic shows antiviral activity on vaccinia and herpes simplex viruses \textit{in vitro} or in experimental infections \cite{60-64}.

Distamycin A encapsulated nanogels were synthesized using the following method:

TMOS (sigma > 99\%) was hydrolyzed using 2mM HCl for 40 minutes, 2TMOS:1HCl. Distamycin A (Sigma Aldrich) was mixed in potassium phosphate buffer pH 7.5 50mM. This solution was added to the hydrolyzed TMOS solution (1:1 ratio) resulting in a sol-gel block. Distamycin A was incorporated inside the sol-gel matrix during gelation. The sol-gels produced were dried at room temperature for 12 hours, slightly crushed using a spatula and lyophilized for 5 hours. (LABCONCO Free Zone 2.5) (\textbf{Figure 41}).

\textbf{Figure 41.} Illustration of synthesis of Distamycin A encapsulated nanogels.
The incorporation of Distamycin A inside the nanogels was tested by suspending the nanogels in buffer and checking the characteristic UV/Visible absorbance of Distamycin at 303 nm (Figure 42). It was seen that Distamycin A was successfully encapsulated inside the nanogels. Distamycin A being a small molecule readily diffuses out of the nanogels (described in Chapter 3). Nevertheless these studies indicate compatibility of various small molecules with nanogels which show promise for further development.

Figure 42. UV/Vis Spectra of Distamycin A encapsulated nanogels. The spectra shows peak at characteristic wavelength 303 nm indicating encapsulation of Distamycin A inside nanogels.
SILVER NANOPARTICLE – SOL-GEL HYDRID NANOGELS

Resistance of bacteria to bactericides and antibiotics has increased in recent years due to the development of resistant strains. Some antimicrobial agents are extremely irritant and toxic and there is much interest in finding ways to formulate new types of safe and cost-effective biocidal materials. Previous studies have shown that antimicrobial formulations in the form of nanoparticles could be used as effective bactericidal materials. It has been demonstrated that highly reactive metal oxide nanoparticles exhibit excellent biocidal action against Gram-positive and Gram-negative bacteria [65]. Thus, the synthesis and characterization of inorganic particles opens the possibility of formulation of a new generation of bactericidal materials. Various studies have indicated that silver ions and silver-based compounds are highly toxic to microorganisms showing strong biocidal effects on as many as 16 species of bacteria including *E. coli* [65-70]. Thus, silver ions, as an antibacterial component, have been used for various applications such as dental resin composites and ion exchange fibers and in coatings of medical devices [70-73]. The antibacterial activity of silver ions is well known and has been studied in detail. In 2004 Sondi and group have investigated the antibacterial activity of nontoxic elementary silver, in the form of nanoparticles and demonstrated the toxicity of silver towards bacteria [66].

In an effort to prepare a novel safe and nontoxic silver carrier, we have synthesized and characterized hybrid nanogels by incorporating silver nanoparticles inside sol-gel based nanoparticles.
PROCEDURE

Synthesis of Silver Nanoparticles

The solution containing Silver Nanoparticles was prepared in two steps. First 45mg of silver nitrate (AgNO$_3$) was dissolved in 250mL of deionized water and heated slowly until the solution reached to 95-98 °C. In the second step, a 5ml of 1% (w/w) sodium citrate solution was made and added to the heated silver nitrate solution. This mixture was kept between 95 to 98 °C for an hour and product formation was evident when the mixture’s color changed to dark yellow-greenish. After about 1 hour the solution was cooled slowly to room temperature.

Synthesis of Silver nps –Sol-gel Hybrid nanogels

Silver nanoparticle solution was mixed with 50mM pH 7 phosphate buffer in a 1:1 ratio. This mixture was added to hydrolyzed TMOS solution (1:1 ratio). After mixing uniformly the solution formed a sol-gel within 2 minutes. The film was dried at room temperature for 12 hours crushed and lyophilized for 5 hours resulting in hybrid nanogels (Figure 43).

**Figure 43.** Illustration for Synthesis of Silver nanoparticle - sol-gel hybrid nanogels.
The synthesized nanogels were characterized using Dynamic Light Scattering, UV/Visible Spectroscopy and Transmission Electron Microscopy.

Table 7. Effective Diameter and Zeta Potential of Synthesized Hybrid nanogels as characterized using DLS studies. Encapsulation of silver nanoparticles inside the nanogels does not affect the original particle size and colloidal stability of the nanogels.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Effective Diameter (nm)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silver Nanoparticles</td>
<td>50</td>
<td>-50</td>
</tr>
<tr>
<td>Sol-gel nanoparticles (nanogels)</td>
<td>360</td>
<td>-30</td>
</tr>
<tr>
<td>Silver Nanoparticles encapsulated in nanogels (Hybrid nanogels)</td>
<td>370</td>
<td>-30</td>
</tr>
</tbody>
</table>

Silver nanoparticles as seen from TEM and DLS studies are about 50nm in size, they have good colloidal stability with a zeta potential of -50 mV. Encapsulation of silver nanoparticles inside the nanogels does not affect the original particle size and colloidal stability of the nanogels (as seen from the almost similar effective diameter and zeta potential values) (Table 7).
The incorporation of silver nanoparticles inside the nanogel matrix was confirmed by UV/Visible spectroscopy of hybrid nanogels which shows characteristic peak of silver nanoparticles at 420nm (Figure 44).

**Figure 44.** The figure compares UV/Vis spectra of the hybrid nanogels (blue trace); of control nanogels that do not contain silver nanoparticles (red trace) and of the bathing buffer in which the hybrid nanogels were incubated for 24 hours and then separated (black trace).
Conclusions

Various antibiotics were encapsulated inside the nanogels and tested on *E.coli*. The synthesized nanogels were able to kill bacterial cells indicating that encapsulated antibiotics are able to diffuse out from the nanogel matrix in a sustained manner and show therapeutic effect. Control nanogels without any antibiotic show no toxicity towards bacteria confirming the nontoxic properties of nanogel matrix offering a huge advantage as a carrier.

Silver nanoparticles were encapsulated inside the nanogels resulting in hybrid nanogels which are stable and intact and do not change the properties of the nanogel matrix.

Silver nanoparticles are known to have great potential for applications such as biosensors, biological tags for quantitative detection. They also have antibacterial applications and have been incorporated in apparel, footwear, paints, wound dressings, appliances, cosmetics, and plastics. Hybrid nanogels can provide additional benefits by acting as a nontoxic, inert carrier with sustained release from porous sol-gel matrix. Properties of sol-gel such as PEG binding, nonimmunogenic, surface decoration can be utilized if needed.
CHAPTER 7

H₂O₂ AND NO DELIVERY USING NANOGELS
A cell survival study on E.coli

Nitric oxide (NO) is a short-lived, diatomic, lipophilic gas that plays an integral role in defending against pathogens. NO regulates a range of biological processes in the cardiovascular, gastrointestinal, genitourinary, respiratory, and central and peripheral nervous systems [74, 75]. Among its many functions are involvement in immune cell signaling and in the biochemical reactions by which immune cells defend against bacteria, fungi, viruses and parasites. NO signaling directs a broad spectrum of processes, including the differentiation, proliferation, and apoptosis of immune cells. Furthermore, the discoveries of NO as a vasodilator, antibacterial agent, and tumoricidal factor have made NO a promising pharmaceutical agent [74-78]. These observations led to the need for NO delivery systems that can harness its antimicrobial properties. A number of synthetic compounds that chemically store and release NO in a controlled fashion have been developed. Several classes of NO donors exist including nitrosothiols, nitrosamines, diazeniumdiolates, NO-metal complexes, and organic nitrites and nitrates [78,79].

Sodium Nitroprusside (SNP) is one such NO donor. Nitroprusside is a complex anion that features an octahedral ferrous center surrounded by five tightly bound cyanide ligands and one linear nitric oxide ligand (Figure 45). SNP when exposed to light generates NO [79-82]. Since the sol-gel is a glass like transparent substance it is apt for encapsulation of photosensitive materials which can then be controlled to generate desired products.
The glucose oxidase enzyme (GOx) is an oxido-reductase that catalyzes the oxidation of glucose to hydrogen peroxide and D-glucono-δ-lactone. In cells, it helps in breaking sugar down into its metabolites. GOx (often extracted from Aspergillus niger) is widely used for the determination of free glucose in body fluids (diagnostics), in vegetal raw material, and in the food industry. It also has many applications in biotechnology, typically enzyme assays for biochemistry including biosensors in nanotechnology [28].

Our studies have showed (Chapter 5) that GOx when encapsulated inside the sol-gel matrix is functionally active and is able to generate H₂O₂ when glucose is provided. The antimicrobial properties of H₂O₂ have been studied in depth and are well known. Previous studies by some groups and experiments done by us have demonstrated that Nitric oxide potentiates hydrogen peroxide-induced killing of Escherichia coli [83, 84] (Figure 46). For prokaryotes subjected to oxidative injury, NO was found to enhance the bactericidal effect of H₂O₂, lending support to the concept that NO plays a critical role in host defense against invading pathogens. It has also been demonstrated in our studies that the presence of cyclic nitrooxide antioxidants such as TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl) (Figure 46,47) protect the cells from the synergic killing effect [83].
**Figure 46.** *E.coli* cell survival studies showing the synergistic activity of NO and H\(_2\)O\(_2\) in solution. H\(_2\)O\(_2\) alone shows some killing, however NO markedly potentiates H\(_2\)O\(_2\)-induced killing of *E. coli*. The antioxidant TEMPOL abrogates the synergic killing effect of NO and H\(_2\)O\(_2\).

**Figure 47.** Structure of the antioxidant 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL).
Based on our previous experiments the suggested mechanism for the NO and H₂O₂ synergic killing effect is described below.

A main route for H₂O₂ toxicity is through the formation of OH radicals (Hydroxyl radicals). This reaction is very slow but is catalyzed by iron, through the Haber-Weiss reaction. (See scheme below). In the cells, nitric oxide can release Iron ions from bacterial [4Fe-4S] protein clusters storage, thereby providing the catalyst for the Haber-Weiss reaction, forming OH radicals and resulting in a synergic killing effect. Moreover, nitroxides such as TEMPOL can effectively oxidize cellular redox-active metals and pre-empt the Fenton reaction explaining the abrogation of the synergic killing effect in the presence of TEMPOL [83].

<table>
<thead>
<tr>
<th>The Haber-Weiss Catalytic Cycle:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Step I.</strong> Reduction of ferric ion to ferrous ion</td>
</tr>
<tr>
<td>Fe³⁺ + •O₂⁻ → Fe²⁺ + O₂</td>
</tr>
<tr>
<td><strong>Step II:</strong> The Fenton Reaction</td>
</tr>
<tr>
<td>Fe²⁺ + H₂O₂ → Fe³⁺ + OH⁻ + •OH</td>
</tr>
<tr>
<td>Net reaction:</td>
</tr>
<tr>
<td>•O₂⁻ + H₂O₂ → •OH + OH⁻ + O₂</td>
</tr>
</tbody>
</table>

Combination of H₂O₂ and NO is highly toxic to prokaryotes and has potential in antimicrobial therapy. However maintaining a constant H₂O₂ concentration for treatment becomes complicated because microorganisms have their own biological mechanisms for removal of H₂O₂ (using cellular catalases and peroxidases). To overcome this problem a sustained release of H₂O₂ and NO is required for treatment which will be able to continuously
deliver the drugs to the bacterial cells. We have synthesized antibacterial, NO and H$_2$O$_2$ generating nanogel delivery systems, by encapsulating the enzyme glucose oxidase (GOx) and the NO donor SNP inside the nanogels. These nanogels when exposed to dextrose and light will generate H$_2$O$_2$ and NO simultaneously in a controlled manner (Figure 48). We have also demonstrated that TEMPOL encapsulated nanogels protect bacterial cells from synergic killing by H$_2$O$_2$/NO combination. Herein, we report a biocompatible mild synthetic approach to preparing NO and H$_2$O$_2$ generating nanogels which have the potential to act as treatment for combination therapy for multidrug resistant bacteria.
Figure 48. Illustration shows a combination of GOx and SNP nanogels, which when exposed to Glucose and Light generate NO and H₂O₂ which are released into the surrounding solution.
**Procedure**

**Synthesis of NO generating nanogels.**

TMOS (sigma > 99%) was hydrolyzed using 2mM HCl for 30 minutes (Branson). Potassium Phosphate Buffer pH 7 50mM (with desired SNP concentration) was added to the hydrolyzed TMOS solution (1:1 ratio). SNP was incorporated inside the sol-gel matrix during gelation. The sol-gels produced were dried at room temperature for 12 hours, slightly crushed using a spatula and lyophilized for 5 hours (LABCONCO Free Zone 2.5).

**Synthesis of H$_2$O$_2$ generating nanogels.**

TMOS (sigma > 99%) was hydrolyzed using 2mM HCl for 30 minutes (Branson). Potassium Phosphate Buffer pH 7 50mM (with desired GOx concentration) was added to the hydrolyzed TMOS solution (1:1 ratio). GOx was incorporated inside the sol-gel matrix during gelation. The sol-gels produced were dried at room temperature for 12 hours, slightly crushed using a spatula and lyophilized for 5 hours. (LABCONCO Free Zone 2.5)

**Generation of H$_2$O$_2$ and NO from nanogels.**

The generation of H$_2$O$_2$ was measured using an iodide assay and UV/Visible spectroscopy (absorbance at 352 nm). The generation of NO was indirectly measured by nitrite determination using griess’s reagent. (Absorbance at 540nm) (thermo scientific nanodrop2000).
Dynamic Light Scattering and Zeta Potential Analysis

DLS measurements were performed on a 90 plus particle size analyzer (Brookhaven Instruments Corporation) employing a 90° scattering angle and a 35 mW incident laser (658 nm). Small amounts of nanogels (1-2mg) were suspended in deionized water and sonicated for 15 minutes before taking measurements. The reported diameter is the average of 5 runs for each sample.

Zeta Potential measurements were done by adding small amount (1-2mg) of nanogels in 1mM KCl solution and sonication for 15 minutes (Brookhaven Instruments Inc. Zeta Plus).

Transmission Electron Microscope (TEM) Studies

Dilute samples used for DLS measurements were mixed in a 1:1 ratio with 1% uranyl acetate, sonicated for 10 minutes. Then 5ul was put on a carbon film coated copper grid, 300 mesh (electron microscopy sciences, Hatfield, PA) and the excess solution was removed immediately using absorbent paper. The grids were dried overnight and viewed under the transmission electron microscope (JEOL, model JEM-1200 EX). Images were taken with a SIA–L3C CCD camera (Scientific Instruments and Applications, Inc.) using the software Maxim DL5 (Diffraction Limited, Ottawa, Canada).

E.coli experiments

E. coli was grown aerobically at 37°C for 12-16 h with shaking (250 rpm) in Luria-Bertani medium (LB). The saturated cultures were diluted in fresh LB medium starting at (OD 600) 0.05 (optical density) and incubated at 37 °C in Erlenmeyer flasks with vigorous shaking at 250 rpm as well until they reached a concentration of 2x10^5 cells/ml. Each E.coli solution was incubated with different nanogels for a total time of 90 minutes. All samples were exposed to light and dextrose at time 0. Catalase (10U/ml) was added to remove residual H2O2 and terminate the
oxidative stress. Four dilutions were made at each time interval and three 20 ul drops were plated from each dilution on solid agar plates. Plates were incubated overnight at 37°C and colonies counted. All results were compared to the control without any nanogels.

**Results**

DLS measurements showed that the effective diameter of the particles produced was between 100-500 nm (Table 8). GOx and SNP nanogels are also colloidal stable as seen by the zeta potential of -35 and -31mV respectively (Table 9).

**Table 8.** Size of GOx and SNP nanogels as measured by Dynamic Light Scattering.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Effective Diameter (nm) (from DLS)</th>
<th>Size (nm) (from TEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control nanogels</td>
<td>428</td>
<td>60-100</td>
</tr>
<tr>
<td>GOx encapsulated nanogels</td>
<td>294</td>
<td>20-100</td>
</tr>
<tr>
<td>SNP nanogels</td>
<td>259</td>
<td>20-100</td>
</tr>
</tbody>
</table>

**Table 9.** Zeta Potential of GOx and SNP nanogels as measured by the Zeta Analyzer.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control nanogels</td>
<td>-30</td>
</tr>
<tr>
<td>GOx encapsulated nanogels</td>
<td>-35</td>
</tr>
<tr>
<td>SNP nanogels</td>
<td>-31</td>
</tr>
</tbody>
</table>
Transmission electron micrographs show (Figure 49) that the nanogels are circular, well distributed particles in the size range of 20-100nm.

Figure 49. TEM micrographs of a. SNP encapsulated nanogels, Scale Bar: 0.5µm; b. GOx encapsulated nanogels, Scale Bar: 0.5 µm. The average size of the nanogels for both samples range from 20-100nm.
GOx and SNP were successfully incorporated inside the nanogels. As tested by UV visible spectroscopy it was seen that the GOx is not only successfully encapsulated within the sol-gel matrix, but is also functional as it is able to generate hydrogen peroxide upon addition of glucose to the nanogel solution. The generation of H$_2$O$_2$ was spectrophotometrically measured using an iodide assay (wavelength 352nm) (Figure 50a). When the nanogels are suspended in a solution of glucose, the glucose molecules are able to travel inside the porous matrix and react with the enzyme to generate H$_2$O$_2$, which is released out into the solution.

Similarly, SNP encapsulated nanogels when exposed to light generate NO (NO is quickly converted to nitrite which can be measured by Griess’s reagent at wavelength 540nm) (Figure 50b).

Figure 50. a. Generation of H$_2$O$_2$ from GOx encapsulated nanogels upon exposure to glucose. The concentration of H$_2$O$_2$ at each time point was determined using an iodide assay (352nm); b. Generation of Nitrite from SNP encapsulated nanogels upon exposure to light. The concentration of Nitrite at each time point was determined using Griess reagent (540nm).
**Generation of H$_2$O$_2$ and NO from nanogels synthesized using Lyophilization protocol.**

H$_2$O$_2$ and NO generating nanogels were tested on cell survival of bacteria. During the experiments there was continuous monitoring of the concentrations of H$_2$O$_2$ and NO concentration using the iodide assay and Griess reagent respectively.

The results demonstrated that GOx nanogels are able to show killing towards *E.coli*. Moreover the combination of GOx nanogels and SNP nanogels show much higher killing rate as compared to GOx alone. This effect is due to the H$_2$O$_2$/NO release combination (Figure 51). Using nanogels we were able to demonstrate the same effect as seen in solution indicating that nanogels are simultaneously able to deliver H$_2$O$_2$ and NO.

When the GOx and SNP is encapsulated inside the same nanogels, the same combination of H$_2$O$_2$/NO toxicity is seen again (Figure 52). These results show the superior property of nanogels as a hybrid or multipurpose carrier.
Figure 51. a. Growth curves of *E.Coli* when incubated with different nanogels. GOx nanogels are able to show killing towards *E.coli*. Moreover the combination of GOx nanogels and SNP nanogels show much higher killing rate as compared to GOx alone. This effect is due to the H$_2$O$_2$/NO release combination; b. Concentration of nitrite as a function of time during the experiment (measured using Griess reagent); c. Concentration of H$_2$O$_2$ generated as a function of time during the experiment (measured using iodide assay).
**Figure 52.** Growth curves of *E. Coli* when incubated with hybrid nanogels containing all the chemicals in one nanogel matrix. When the GOx and SNP is encapsulated inside the same nanogels, the same combination H2O2/NO toxicity is seen again.
It was seen in solution experiments that TEMPOL is capable of protecting the bacterial cells from the H₂O₂/NO synergistic killing. When TEMPOL encapsulated nanogels are added to the combinations of GOx ngs and SNP ngs, the TEMPOL is able to diffuse put and protect the cells from killing (Figure 53).

**Figure 53.** *E.coli* cell survival study showing protection of *E.coli* cells from H₂O₂/NO toxicity by TEMPOL encapsulated nanogels.

**Generation of H₂O₂ and NO from nanogels synthesized using Polylsine protocol.**

Generation of H₂O₂ and NO was also achieved using nanogels produced using the Polycationic peptides. The nanogels were able to continuously generate H₂O₂ and NO and were able to show killing towards *E.coli* cells (Figure 54).
Figure 54. a. Growth curves of E.Coli when incubated with H₂O₂ generating nanogels synthesized using the polylysine protocol, SNP was added externally; b. Concentration of nitrite as a function of time during the experiment (measured using Griess reagent); c. Concentration of H₂O₂ generated as a function of time during the experiment (measured using Iodide assay).
Nanogels synthesized using both protocols (Lyophilization and Polylysine protocol) displayed similar results. When *E.Coli* was incubated with GOx encapsulated nanogels, the growth curves showed substantial killing of the bacteria over time as compared to control bacteria, which was correlated to the H$_2$O$_2$ generated from the GOx nanogels (measured simultaneously). Moreover a combination of GOx encapsulated nanogels and SNP encapsulated nanogels displayed enhanced killing of the bacteria due to the potentiating effect of the NO released towards the killing effect of H$_2$O$_2$.

**Conclusions**

This study shows that enzymes and small molecules co-encapsulated inside the nanogel matrix are capable of carrying out multiple reactions and generate products in a sustained manner. We were able to reproduce results from H$_2$O$_2$/NO synergy solution studies inside nanogels. The H$_2$O$_2$ and NO generating nanogels that were studied here displayed good antibacterial activity against *E.coli*. This work integrates nanotechnology and bacteriology, leading to possible advances in the formulation of new types of bactericides. However, future studies on the biocidal influence of this nanomaterial on other Gram positive and Gram-negative bacteria are necessary in order to fully evaluate its possible use as a new bactericidal material.
CHAPTER 8

OUTLOOK

Important Features of Sol-Gel Based Nanoparticles

One of the most useful properties of sol-gel based nanoparticles is easy and mild synthetic technique. There is minimal effect of the silicate matrix on the properties of encapsulated therapeutic molecules and macromolecules and they remain effective and functional. Successful co-encapsulation of different biologically active agents can be achieved for multi targeted therapy. Our studies have shown sustained delivery of sol-gel co-encapsulation of therapeutic agents to bacterial cells. Moreover the nanogels in themselves exhibited a very low toxicity towards bacteria. All these properties are highly desirable in a carrier matrix for therapeutic agents and show promise for further development.

Future Development

We have in this work developed recipes to easily synthesize nanogels and have characterized some aspects of these particles that will be useful for further development of them as therapeutic carriers. There are still interesting questions which are worthwhile to explore, and further developments are necessary. It would also be interesting to conduct studies to find different silica precipitating agents to improve biomimetic methods for production of non-toxic and inert nanoparticles. Further investigations into effect of these nanogels on different kinds of microorganisms would be useful for advancement of such nanoparticles for antimicrobial therapy. Although there have been some studies on intake of silicate nanoparticles by mammalian cells, interactions between nanogels and cells are not fully understood and need
further probing. Studies in mammalian cell cultures are needed. Assessment of bio-distribution and blood circulation levels and interaction with serum proteins need to be studied in detail and testing in animal models is required for further development of nanogels for therapeutic purposes.

**Potential Applications**

Nanogels offer potential for various kinds of interesting applications which we came across as we developed methods to synthesize and characterize them. An important application is Multidrug resistance antibiotic therapy for infections such as Methicillin-resistant *Staphylococcus aureus* (MRSA). Combination therapy using various antibiotic agents would be compatible with the nanogel carrier as shown in our studies. Since the nanogel matrix can hold proteins intact and unharmed, it has potential to be used in diagnostics and biosensor fields. Further developments into surface modification of the nanogels would also enable them to be used as inert, nontoxic agents for potential antitumor therapy.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APTES</td>
<td>3-Amino propyl triethoxy silane</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>DTT</td>
<td>Sodium Dithionite</td>
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<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared spectroscopy</td>
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<tr>
<td>GOx</td>
<td>Glucose Oxidase</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
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<tr>
<td>Mb</td>
<td>Myoglobin</td>
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<tr>
<td>ngs</td>
<td>Nanogels</td>
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<tr>
<td>NO</td>
<td>Nitric Oxide</td>
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<tr>
<td>nps</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
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<tr>
<td>PLL</td>
<td>Poly-L-Lysine</td>
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<tr>
<td>SNP</td>
<td>Sodium Nitroprusside</td>
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<tr>
<td>Soln.</td>
<td>Solution</td>
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<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
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<tr>
<td>TEMPOL</td>
<td>4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl</td>
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<tr>
<td>Tf</td>
<td>Transferrin</td>
</tr>
<tr>
<td>THPMP</td>
<td>3-(trihydroxysilyl) propyl methyl phosphonate</td>
</tr>
<tr>
<td>TMOS</td>
<td>Tetra methyl orthosilicate</td>
</tr>
<tr>
<td>TQ</td>
<td>Thymoquinone</td>
</tr>
<tr>
<td>UV/Vis</td>
<td>UV visible spectroscopy</td>
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BIBLIOGRAPHY


