SOL-GEL SYNTHESIS OF DNA ENCAPSULATED SILICA

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SOL-GEL SYNTHESIS OF DNA ENCAPSULATED SILICA

Submitted by **DERYA KAPUSUZ** in partial fulfillment of the requirements for the degree of **Master of Science in Metallurgical and Materials Engineering Department, Middle East Technical University** by,

Prof. Dr. Canan ÖZGEN
Dean**, Graduate School of Natural and Applied Sciences** _____

Prof. Dr. Tayfur ÖZTÜRK
Head of Department, **Metallurgical and Materials Engineering** _____

Assoc. Prof. Dr. Caner DURUCAN
Supervisor, **Metallurgical and Materials Engineering Dept., METU** _____

Examining Committee Members:

Prof. Dr. Meral YÜCEL _____
Biological Sciences Dept., METU

Assoc. Prof. Dr. Caner DURUCAN
Metallurgical and Materials Engineering Dept., METU _____

Assist. Prof. Dr. F.Arcan DERİCİOĞLU
Metallurgical and Materials Engineering Dept., METU _____

Assist. Prof. Dr. Burcu AKATA KURÇ
Micro and Nanotechnology Dept., METU _____

Assist. Prof. Dr. Dilek KESKİN
Engineering Sciences Dept., METU _____

Date: 05.06.2009

I hereby declare that all information in this document has been obtained and presented accordance with the academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name : Derya KAPUSUZ

Signature :

ABSTRACT

SOL-GEL SYNTHESIS OF DNA ENCAPSULATED SILICA

Kapusuz, Derya

M.Sc., Department of Metallurgical and Materials Engineering

Supervisor: Assoc. Prof. Dr. Caner Durucan

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Sol-gel processing routes for encapsulation of double stranded DNA in solid porous silica hosts have been established. The encapsulation was carried out in two steps: hydrolysis of a silica-forming alkoxide-based sol was followed by condensation/gelation to a solid form upon addition of a buffer solution containing DNA molecules. The effects of gelation chemistry and DNA amount on chemical and microstructural properties of resultant silica matrices and on DNA encapsulation efficiency were investigated. The analytical characterization was performed by UV-vis spectroscopy, ^{29}Si nuclear magnetic resonance spectroscopy and by nitrogen adsorption studies. It was demonstrated that DNA incorporation had a pH-dependent catalytic effect on gelation kinetics and promoted silica network completion. In addition, the scale of porosity and the average pore size of the resultant silica increased with gelation pH and also with DNA-buffer solution in the starting sol-gel formulation. The chemistry-derived pore size variation controls the DNA encapsulation efficiency in the silica matrices and the DNA holding capacity strongly depends on the scale of the porosity attained.

The selective adsorption of ethidium bromide- a DNA-intercalative reagent molecule- on DNA-silica gels confirmed that the DNA molecules remained entrapped within the silica host in their native state without any deterioration. Besides pure silica, amine-functionalized hybrid silica hosts were also formed by sol-gel. The hybrid gels were found not to be suitable for DNA encapsulation, as these matrices dissolve in aqueous environment due to incomplete silica network formation. The DNA-doped silica hosts may provide promising matrices for development of biosensors, bioreactors and bioassay platforms.

Keywords: silica, DNA, sol-gel, pore size, encapsulation

ÖZ

SOL-JEL YÖNTEMİYLE HAZIRLANMIŞ SİLİKA MATRİSLERDE

DNA ENKAPSULASYONU

Kapusuz, Derya

Y.Lisans, Metalurji ve Malzeme Mühendisliği Bölümü

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Sol-jel üretim süreçleri kullanılarak çift zincirli DNA molekülü gözenekli katı silika yapılar içinde enkapsule edilmiştir. Enkapsulasyon iki aşamada gerçekleştirilmiştir; ilk olarak hidrolize edilen alkoksit-esaslı silika kaynağı daha sonra DNA içeren tampon çözeltinin eklenişi ile yoğunlaşarak jelleştirilmiştir. Jelleşme kimyasının DNA enkapsulasyon verimine ve silika mikroyapısal özelliklerine olan etkileri araştırılmıştır. DNA enkapsulasyon verimi ve silika matris özelliklerini belirlemek üzere UV-vis spektroskopisi, nükleer manyetik rezonans spektroskopisi ve nitrojen adsorpsiyonu teknikleri kullanılmıştır. DNA eklenişinin jelleşme üzerinde hızlandırıcı etkisi olduğu ve silika ağ yapısı oluşumunu arttırdığı gözlenmiştir. Ek olarak, sol-jel formulasyonundaki DNA miktarına bağlı olarak pH arttıkça ortalama gözenek boyutunda artış meydana gelmiştir. Jelleşme kimyasına bağlı olarak değişen gözeneklilik ve ortalama gözenek boyutunun silika matrislerinde DNA enkapsulasyon verimini belirleyen başlıca faktörler olduğu tespit edilmiştir.

Etidiyum bromid interkalasyon testleri DNA moleküllerinin silika yapısı içinde bozunmadan enkapsule edildiğini göstermiştir. Öte yandan, silika oluşumu ve DNA enkapsulasyon verimi üzerinde amin katkılı hibrid modifikasyonunun etkisi araştırılmıştır. Bu hibrit jellerde silika ağ-yapısı oluşumu katkısızlara göre daha düşük oranda gerçekleşmiştir. Elde edilen sonuçlar hibrit yapıda üretilen silika jellerin su içerikli ortamlara dayanıklı olmaması sebebiyle DNA enkapsulasyonu için uygun olmadığını göstermiştir. Bahsi geçen DNA içerikli silika jelleri biyolojik sensör, reaktör ve analiz platformlarında kullanım potansiyeli taşımaktadır.

Anahtar Kelimeler : silika, DNA, sol-jel, gözenek boyutu, enkapsulasyon

To my mom...

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LIST OF ABBREVIATIONS

A_{260} : UV-vis absorption at 260 nm

APS : 3-Amino-prophyl triethoxy silane, $SiC_3H_6NH_2(OC_2H_5)_3$

BET method: Branuer-Emmet-Teller method

$DNA_{[TE]}$: DNA in TE buffer

EtBr : Ethidium bromide, $C_{21}H_{20}BrN_3$

EtOH : Ethanol, C_2H_5OH

^{29}Si NMR Spectroscopy: Silicon Nuclear magnetic resonance spectroscopy

TEOS : Tetraethyl orthosilicate, $Si(OC_2H_5)_4$

TE : Tris-EDTA

UV-vis : Ultraviolet-visible

CHAPTER 1

INTRODUCTION

1.1 Biomolecule immobilization in/on solid materials

Survival of the cells and the continuity of the biochemical reactions in organisms mainly depend on a genetically determined program as realized by the biomolecules such as DNA, RNA and proteins. Critical capabilities of these biomolecules have brought the idea of immobilization and stabilization of them in/on solid materials. It is very difficult to control the structure and stability of those of nano/micro sized biological molecules, thus any novelty in this branching field of nanobiotechnology would have a tremendous effect on the improvement of many different applications with successful use of different biomolecules. The applications of bio-doped materials may include bio-sensing devices, assay platforms, stationary phases for chromatographic analyses, controlled release agents, solid phase bio-catalysts and drug delivery systems [Klibanov (1983), Ellerby et al. (1992), Dave et al. (1994), Bhatia and Brinker (2000), Avnir et al. (2006)]. In addition, they can be also used for the detection of warfare agents for sensing platforms or for bio-separation processes depending on the function of the stabilized biomolecule [Kandimalla et al. (2006)]. The utilization of these applications mostly exacts the integration of microelectronics with biology. Thus, the development of such biocomposites with long term stability of immobilized species inside is a real challenge for materials scientists.

1.2 Background information and literature review

1.2.1 Methods of biomolecule immobilization in/on synthetic materials

Immobilization of biomolecules in/on synthetic materials has been studied by researchers for couple decades. It is not possible to define a single immobilization approach applicable for all biomolecules since they differ in their structural properties. There are different methods available in the literature.

Immobilization methods can be classified in two main groups which are *(i) chemical* and *(ii) physical immobilization* methods. As the names imply, chemical immobilization techniques involve chemical bonding between the host and biomolecule while physical one does not. In the first group, depending on the chemical affinities of the biomolecules and the solid material and possible chemical interactions between them, attachment occurs via covalent bonding. On the other hand, physical immobilization methods involve physisorption in/onto the solid surfaces or physical entrapment (encapsulation). In physisorption, attractions between the biomolecule and the material surfaces are weak. Interactions may be through hydroxyl ions, electrostatic interactions, van der Waals bonding, or through cross linking. In encapsulation process, there is not a chemical interaction between the biomolecule and the material where biomolecule is physically confined in a porous network. The working principles of different immobilization methods are schematically shown in Figure 1.1.

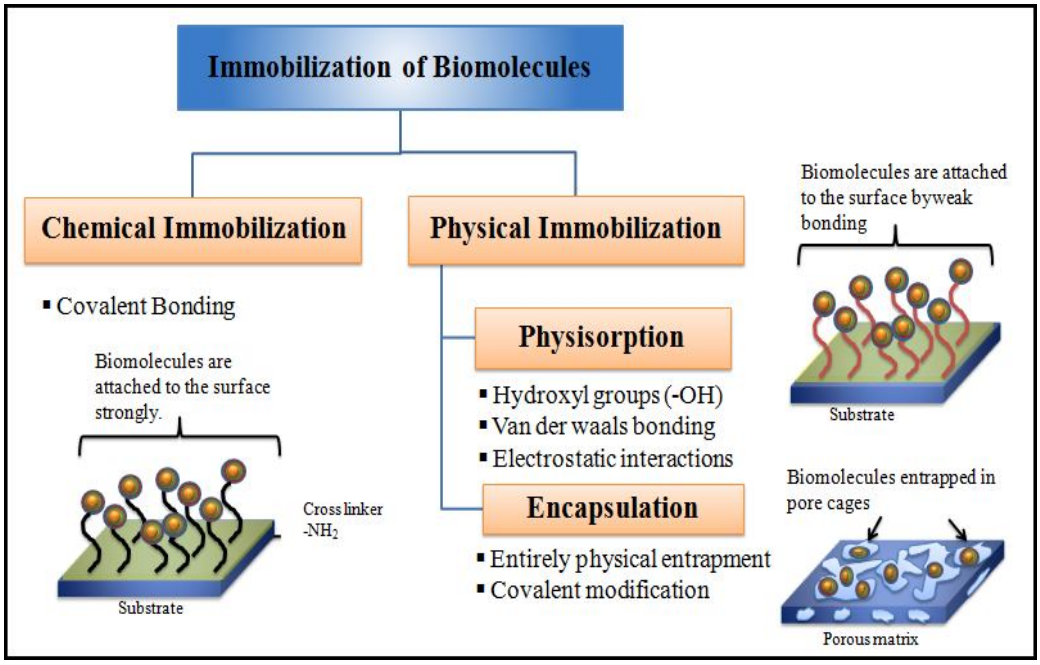

Figure 1.1 Classification and schematics of biomolecule immobilization strategies

1.2.1.1 Chemical immobilization of biomolecules

Covalent bonding is mainly used for biomolecules bound onto the surfaces without being adsorbed or adsorbed with improper conformation. The covalent bonding is chemically strong and the use of this method is very effective to avoid leaching [Gupta and Chaudhury (2007)]. By using suitable linker moieties or by adding chemical functions, it is possible to bind biomolecules onto the polymeric or metallic surfaces. Epoxy resins, polyvinyl plastics and silicone are the most common polymers that have been used for the immobilization of protein molecules [Gill (2001), Gill and Ballesteros (2000)]. The process involves the attachment of enzymes like lipase, esterase and protease to the surface simultaneously with polymerization reaction of the suitable precursor.

Epoxy based, enzyme doped biocomposites have been formed by this method by using precursors like glycidyl-silica or diglycidyl ether. It is also possible to make hybrids with varying hydrophobicity by changing the epoxy precursor and/or the catalyst. However, this type of biocomposites form only surface attached immobilization matrices allowing easier surface leaching. In addition, whatever the matrix polymer is, since the polymerization conditions are too harsh for the biomolecule stabilization, the technique can be utilized to limited types of proteins. As mentioned by Cosnier, electrochemical deposition may be an alternative method for immobilization of biomolecules [Cosnier (1999)]. Electrochemical deposition of biomolecules on polymers with varying thicknesses is a reproducible method and facilitates macromolecular interactions. In electrochemical deposition method, a polymer coating is generated on a small electrode surface and after that covalent attachment of the biomolecule is achieved in its own buffer solution by chemical grafting. Thus, biomolecule stabilization is provided and system does not require any chemical additive which may denature the biomolecule. However, this approach can only provide limited amount of biomolecule attachment at the polymer solution interface in the form of a monolayer.

On the other hand, metallic and inorganic surfaces like gold, silicon or diamond can be also used for anchoring biomolecules. For example, Si-C bonds formed on the hydrogen terminated silicon surfaces allow the covalent immobilization of biomolecules including nucleic acids, peptides and saccharides. For metallic surfaces, the main problem is the degradation of the interface. As an example, Rogero et al. studied the immobilization of DNA molecules on amine modified silicon surfaces [Rogero et al. (2008)]. They stated that, these strategies require an initial attachment of the protected alkyl chains and their subsequent deprotection by acid or base hydrolysis after surface functionalizing.

Alternatively, diamond is another promising host material for biomolecule attachment due to its mechanical strength and chemical inertness. Popova et al. studied RNA and protein (*virp1*) immobilization on diamond nanocrystallites [Popova et al. (2006)]. It was seen that the chemical bonding between the amino group at N terminus of protein and diamond surface coated on silicon surface still necessitated chemical modifications which may cause higher levels of denaturation.

In summary, although covalent bonding of biomolecules onto the polymeric or metallic and inorganic surfaces is strong and provides a proper way for stabilization on the surfaces, such an approach is limited to biomolecules which are in simple conformations. In addition, it is not possible to obtain structurally controlled monolithic biomolecule-solid material systems by covalent attachment. Surface pretreatments and modifications may also cause biomolecule denaturation.

1.2.1.2 Physical immobilization of biomolecules

1.2.1.2.1 Physisorption

Physisorption is another approach for immobilization of biomolecules. It is commonly used due to its simplicity, low cost and potential in maintaining high catalytic activity of enzymes. The necessity of chemical modification and the surface degradation of the material are limited when compared to covalent bonding methods. Due to its less complicated chemistry, it is one of the most commonly used immobilization techniques. It is preferred for the complex structured biomolecules in which the major factor for activity is the shape of the biomolecule, i.e. its conformation.

Metallic, glass or diamond surfaces can be used to attach biomolecules onto those of surfaces by physisorption. For instance, very high surface area of the nanosized diamond crystallites offer surface modifications with carboxyl and amino groups. Also, the chemical treatments with oxidative acids make those surfaces highly sensitive to biomolecule structures [Nguyen et al. (2007)]. But unfortunately, due to the weak chemical attraction, resistance of the surface to leaching threats is very low.

Alternative to crystalline surfaces, sol-gel derived porous amorphous matrices can be also used as "physisorption media". Gao et al. studied *lipase* adsorption onto methyl modified silica aerogels [Gao et al. (2008)]. They basically impregnated the enzyme to subsequently produced silica aerogels and concluded that hydrophobic silica aerogel surfaces were appropriate for the physical adsorption of *C. Rugosa Lipase*. The protected activity was around 55%.

Impregnation- being the main mechanism of physisorption- can be also employed by the formation of sol-gel thin films on glass electrode surfaces. Sahney et al. in their study used this method and produced a silica thin film by dip coating [Sahney et al. (2006)]. They impregnated the *urease* enzyme stock solution after complete drying of the film. Due to the weak interactions between the urease enzyme and the sol-gel derived matrix, enzymes leached from the surface. Also, the diffusion of biomolecules to deeper parts was not controllable and mostly restricted to outer surfaces. Mechanism of physisorption by sol-gel derived materials can be seen in Figure 1.2.

Although physical immobilization techniques offer higher stability of biomolecules, the mechanism of immobilization through processing is not controllable. There are two major difficulties. First, the diffusion is mainly restricted to the surface of the material and additionally, the chemical interactions are so weak that surface leaching becomes a problem whether biomolecules are attached to crystalline surfaces by weak bonding or they are physisorbed.

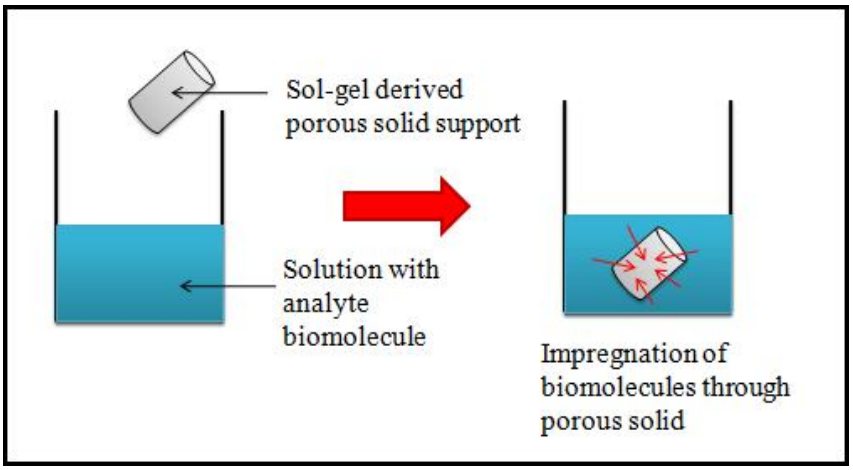

Figure 1.2 Mechanism of biomolecule physisorption (impregnation) into sol-gel derived materials.

1.2.1.2.2 Encapsulation

Unlike chemical immobilization approaches, encapsulation offers stabilization of biomolecules by physical entrapment in materials. As the name implies, the mechanism is the confinement of the biomolecule in a solid material. Several membranes like cellulose membrane, polymeric matrices like polyacrylamide, starch, nylon or sol-gel derived glassy matrices have been studied for encapsulation.

In addition to membranes and polymeric matrices, single walled carbon nanotubes (*SWNT*) and hallow nanospheres have been also reported as encapsulation materials. SWNTs are promising for encapsulation of DNA molecules. By using a plasma ion irradiation method, the structural weakness of DNA molecules can be protected by those of nanotubes. However, it was shown by Kaneko et al. that applied electric field during encapsulation process may change the conformation of DNA in the electrolyte and causes irradiation of the DNA strand to the anode [Kaneko et al. (2007)]. But, DNA conformation is sensitive to electric field so that the encapsulation matrix is restricted to a limited type of applications.

On the other hand, DNA oligomers can be also encapsulated in cationic surfactants. Bio-doped materials produced by this method are to be used in cell transfection and gene therapy. Even tough the helicity of DNA is protected; these kinds of complexes dissolve in organic solvents such as ethanol and chloroform decreasing the chemical retention of the product [Rosa et al. (2007)]. In another study by Chong and Zhao, microsphere encapsulation approach was examined where alumina and/or silica derived hallow microspheres formed by emulsion polymerization can be used for encapsulation of biomolecules. They used polysulfone microspheres derived by liquid-liquid phase separation to encapsulate DNA oligonucleotides. They mentioned that those microsphere biocomposites had high potential to be used in environmental applications as synthetic matrices to accumulate and remove DNA intercalating toxic/pollutant molecules. However, the process is still limited to applicability of liquid-liquid phase separation method [Chong and Zhao (2003)].

On the other hand, the simplicity, low temperature processability, chemical inertness with tunable porosity, optical transparency and provision of aqueous phase with resistance to microbial effects mark sol-gel encapsulation for the immobilization of many types of biomolecules [Kandimalla et al. (2006)].

Sol-gel encapsulation has been used to immobilize biomolecules for about 40 years. It began with pioneering work of Dickey using alkoxy based precursors to obtain bio-doped silica matrices [Dickey (1955)]. Then it continued with the study of encapsulation of enzymes and proteins. Currently, a variety of complex biomolecules and micro-organisms are under investigation for encapsulation, to be used in biomedical applications like medical diagnosis, drug delivery, biosensors and even in organ transplantation.

As a significant advantage of "encapsulation" with respect to other methods, it is possible to form biocomposites in bulk form as in thin film or coating. It does not necessitate any chemical modification and encapsulation is not limited with the surface. The optical transparency of sol gel derived matrices is the major reason that provides the product utilization in biosenser applications. Additives may be used to in sol-gel based bioencapsulation approaches to increase the resistance to leaching and deformation. Since the interaction is only the physical entrapment in the pores, pore size and distributions are the key parameters for efficient encapsulation.

As it has been proved by many studies, biomolecules are stable in the pores with dimensions similar to their sizes [Dave et al. (1994), Gill and Ballesteros (2000)]. By controlling the fundamental sol-gel components and processing conditions, it is possible to obtain proper encapsulation matrices for different types of biomolecules. As it is the technique used in this thesis, sol-gel technique will be introduced more in detail in coming sections.

Many techniques are available for the immobilization of many types of biomolecules. Since biomolecules differ in size and as it is hard to control the stability and functionality in nano scale, it is not possible to use a single type of matrix suitable for the stabilization of different biomolecules.

Apart from proteins and/or enzymes, nucleic acid doped biocomposites have high potential for using in different biomedical applications. However, nucleic acid encapsulation studies are not very common. They have complex conformations and they necessitate aqueous phases to be stabilized. In addition, the ionicity is also very critical. Although all those difficulties, sol-gel process still promises for those biomolecule types. As it is obvious, sol-gel based processing approaches can satisfy these needs for encapsulation of fragile biomolecules to some extent.

The key factor for the ideal encapsulation in sol-gel derived materials is to understand the biomolecule-matrix interactions in order to establish proper encapsulation routes for the biomolecule that is of interest.

1.2.Fundamentals of sol-gel processing

Sol-gel is a low temperature, aqueous, chemical technique used for synthesis and processing of solid materials at different forms. It is possible to obtain solid matrices in particulate, monolith, fiber, thin or thick film forms. Structural evolution during sol-gel processing can follow these steps; *(i) mixing of the precursors and the catalysts*, *(ii) gelation*, *(iii) aging and drying*. Typically, processing steps are shown in Figure 1.3. The structural changes occur during each step also by schematics in Figure 1.4.

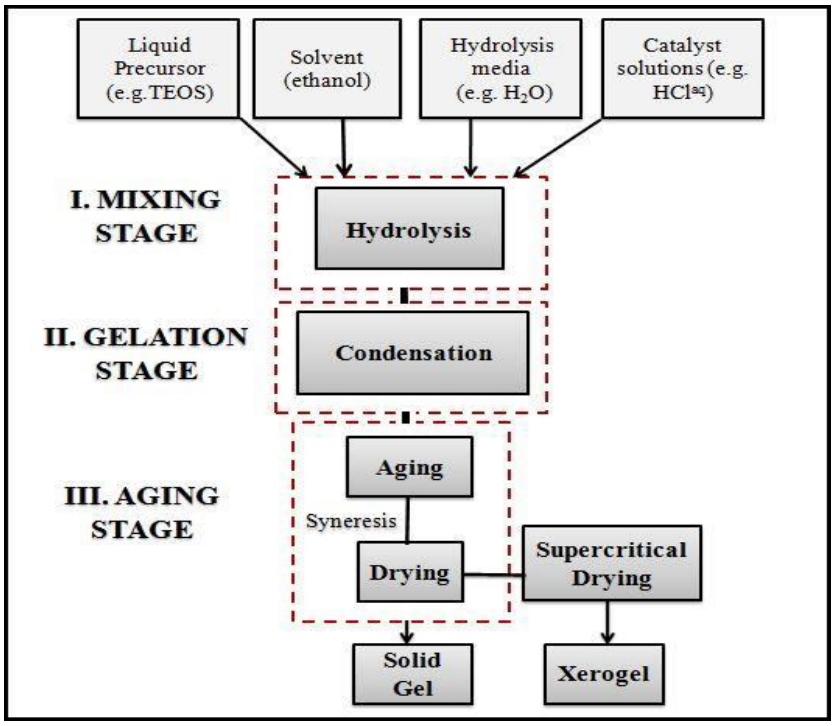

Figure 1.3 Sol-gel processing steps and the constituents for making TEOS derived silica gels.

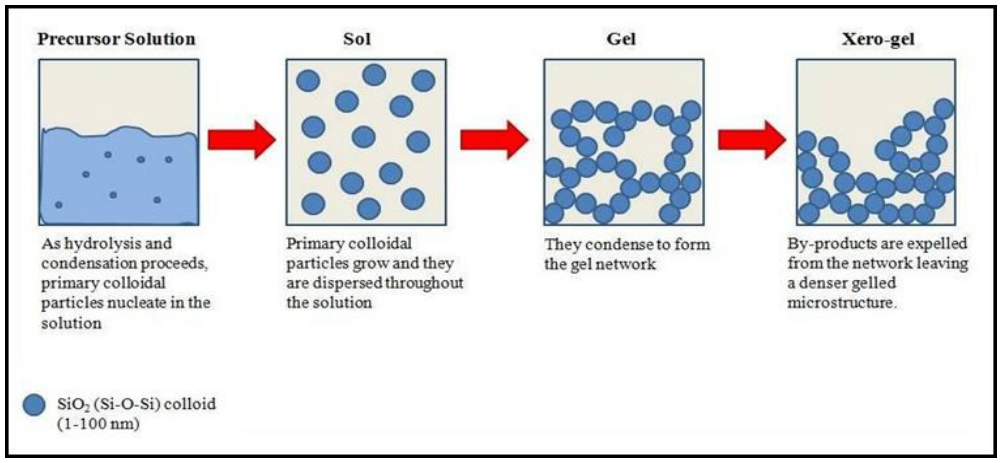

Figure 1.4 Schematics of structural evaluation during sol-gel reactions

1. Mixing: Sol-gel processing initiates with the mixing of the aqueous precursors. Precursors are mainly metal alkoxides or aqueous metal salts. Metal alkoxide precursors are mostly preferred over metallic salts because a careful control of the reaction rate to ensure the structural homogeneity is needed. Metal centers of the precursors may be Si, Al, V, Ti, etc. So, almost any oxide such as SiO_2, Al_2O_3, TiO_2 and V_2O_3 can be synthesized by sol-gel. However, control of chemical reactivity of transition metals is difficult that most studies include the use of silicon alkoxides which are in the form of $Si(OR)_n$ [Brinker and Scherer (1990)]. Silicon alkoxide precursors do not dissolve directly in water, but in organic solvents, mainly alcohols. Then the reactivity differ according to their variable (R) organic group. Longer the organic chain, longer the time needed for the chemical reactions.

In this study, final silica matrix is formed by the gelation of TEOS-based sol (*tetra ethyl orthosilicate, $Si(OC_2H_5)_4$*) because of its superior properties. After the precursors are initially dissolved in suitable alcohols, they react with water molecules forming metal hydroxyl groups leaving their organic chains. This process is called *hydrolysis*. Acid catalyst solutions like acetic acid, hydrogen chloride solutions (HCl) can be used to accelerate the hydrolysis.

After that of formation, at a certain point, previously obtained metal hydroxyl moieties are combined to form the primary particles in which the reaction is called *condensation*. As in hydrolysis, basic catalysts like ammonia solution can be used to accelerate condensation reactions.

As the the degree of network formation increases by time, primary particles poly-condensate to form the secondary particles. General hydrolysis and condensation reactions for an alkoxide precursor are given in Equations (1), (2) and (3) [Jin and Brennan (2002), Gupta and Chaudhury (2007)].

$\equiv M\text{-}OR + H_2O \leftrightarrow \equiv M\text{-}OH + R\text{-}OH$ (*Hydrolysis*) Eq.(1)

$\equiv M\text{-}OR + HO\text{-}M\equiv \leftrightarrow \equiv M\text{-}O\text{-}M\equiv + R\text{-}OH$ (*Alcohol condensation*) Eq.(2)

$\equiv M\text{-}OH + HO\text{-}M\equiv \leftrightarrow \equiv M\text{-}O\text{-}M\equiv + H_2O$ (*Water condensation*) Eq.(3)

Since TEOS precursor was used for producing the silica matrix, sol-gel reactions specific to the production of silica matrices in this study are given in equations (4), (5) and (6).

$\equiv Si\text{-}O(C_2H_5) + H_2O \leftrightarrow \equiv Si\text{-}OH + C_2H_5OH$ (*Hydrolysis*) Eq.(4)

$\equiv Si\text{-}O(C_2H_5) + HO\text{-}Si\equiv \leftrightarrow \equiv Si\text{-}O\text{-}Si\equiv + C_2H_5OH$

(*Ethanol condensation*) Eq.(5)

$\equiv Si\text{-}OH + HO\text{-}Si\equiv \leftrightarrow \equiv Si\text{-}O\text{-}Si\equiv + H_2O$ (*Water condensation*) Eq.(6)

2. Gelation: Gelation takes place when the secondary particles start to agglomerate into a continuous three-dimensional network. This network would also constitute the unreacted species with the by-products, especially alcohols trapped in the aqueous phase in a micro or mesoporous network.

3. Aging and Drying: This step includes the regulation of the microstructure by time. As time passes, since gelation is a kinetic process, before mentioned reactions keep on forming structural units. Remaining by-product alcohols and water are expelled from the pores. This process is called "*syneresis*" in which the pore size becomes smaller due to shrinkage. Since the stresses on the structure are increased due to pore collapsing, some cracks may be formed. In order to obtain crack free surfaces or monoliths, special drying techniques, like supercritical drying, can be used. Gels dried under supercritical drying conditions are termed as "*aerogel*" and the fully dried gels are termed as "*xerogel*".

1.2.2.1 Processing parameters of sol-gel technique

1.2.2.1.1 Precursor type

Final microstructure and properties of sol-gel products depend on several processing parameters. Critical properties that define the microstructural properties of a sol gel derived product are; chemical composition, type and amount of the by-products, gelation rate, mechanical strength and pore sizes and surface area. All of these properties are controlled by combination of various processing parameters. As mentioned before, metal alkoxides, aqueous metals salts or colloidal precursors can be used for the production of porous solid matrices. Since the ionic conditions and the homogeneity are dominant in determining the structural properties, as a disadvantage, it is not easy to control metal salt reactivities under wide pH conditions.

Also while alkoxides can dissolve in organic solvents, the latter cannot [Coradin and Livage (2007)]. All of those disadvantageous properties limit the usage of aqueous metal salts which are most commonly sodium silicates and metal alkoxides are mostly preferred.

Chemical properties of alkoxides differ also between each other. Gelation rate and correspondingly the other properties depend on the length of the "R" organic group i.e, TMOS (*tetramethyl orthosilicate, $Si(OCH_3)_4$*) derived gels are gelled faster than the TEOS (*tetraethyl orthosilicate, $Si(OC_2H_5)_4$*) derived gels.

1.2.2.1.2 H_2O/SiO_2 ratio

Water amount is an effective parameter for the formation and processing of the solid material network. As H_2O/SiO_2 ratio increases, hydrolysis reaction is promoted due to the higher amount of water. When $H_2O/SiO_2<2$, alcohol producing condensation becomes dominant, whereas at $H_2O/SiO_2>2$, water condensation is favored. Higher value of H_2O/SiO_2 ratio allows complete hydrolysis before significant condensation occurs [Gupta and Chaudhury (2007)].

1.2.2.1.3 Catalyst concentration and catalysis mechanism

Many types of acidic and basic catalysts can be used to speed up the hydrolysis and condensation reactions. HCl is one of the most common acid catalysts used to increase the rate of hydrolysis reactions. Use of other acids like acetic acid, nitric acid, KF and HF is also possible. Usually weaker acids require longer reaction times to achieve the same extent of reaction compared to strong acids. Base catalyzed hydrolysis of silicon alkoxides proceeds much more slowly than acid catalyzed hydrolysis at equivalent catalyst concentration.

Bases are used to accelerate the condensation reaction. Most common base catalysts are ammonia and KOH. In addition, two-step catalysis route can be applied to a sol-gel mixture. First the hydrolysis is accelerated by the acid catalyses firstly and then the acceleration of the condensation reaction by basic catalysis. By this way, gelation pH and rate can be controlled. It can be seen from the rate vs. pH graph (Figure 1.5) that fastest gelation can be achieved by optimization of hydrolysis and condensation reactions by two step catalysis route in 4-6 pH range. It must be noted that it is an advantage to have the possibility of controlling the pH around the physiological pH for the usage of biologically active materials within those matrices [Coradin and Livage (2007)].

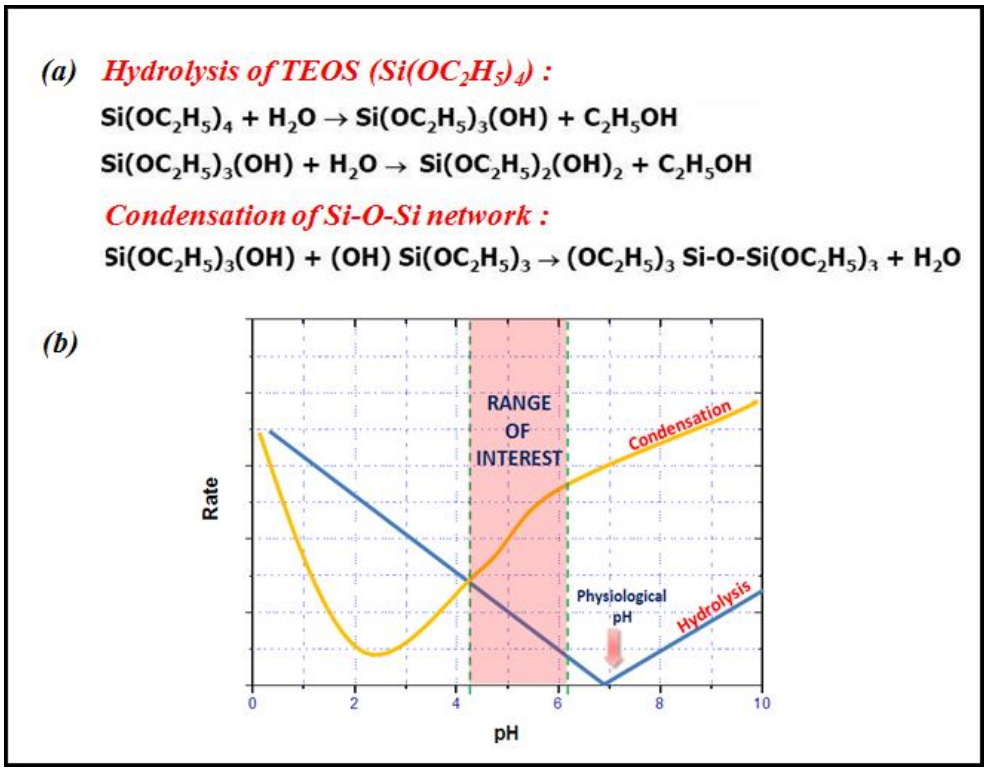

Figure 1.5 (a) Fundamental sol-gel reactions and formation of silica network (Si-O-Si); i) Hydrolysis of TEOS precursor, ii) condensation of silica network,
(b) The relative rates of hydrolysis and condensation reactions during gelation as a function of pH.(*reproduced from Brinker and Scherer (1985))*

1.2.2.2 Effects of processing parameters on porosity

The sol-gel derived final solid materials are porous and pore size and shapes are controlled by above mentioned parameters. Since the subject of this thesis is achievement of encapsulation in porous matrices, parameters affecting the porosity of the pure material structure should be mentioned.

The final pH of the sol-gel derived silica- depending mainly on the catalyst concentration- is the determining factor for porosity. The basic catalysts accelerate the condensation reactions and final microstructures become highly branched. So, when the catalysis routes are compared, it is obvious that not only the mechanisms are different, but also the reaction kinetics is different. For acid-catalysis, hydrolysis is complete and the number of unreacted hydroxyls per silicon decreases with decreasing acid concentration. For base dominant catalysis, the effect of polymerization is large. From this, it is possible to suggest that acid-catalyzed gels have uniform interconnected porosity. Base-catalyzed gels have pores within clusters. Since silicic acid is more soluble in basic solutions, the surface area decreases over time with increasing base and increasing water. With this increased solubility, silica dissolves and reattaches far more easily in basic than acidic aqueous medium increasing pore sizes of the network [Brinker and Scherer (1990)].

1.2.3 Biomolecule encapsulation in sol-gel derived silica matrices

Using the conventional sol-gel process, biomolecules can simply be incorporated to the solid material by replacing the hydrolysis media (water) with buffer solutions carrying the biomolecules or they can be physisorbed to gelled monolith.

Sol-gel technology is known for the past two decades and first enzyme encapsulation in sol-gel silica is studied in early 1950s [Dickey (1955)]. It is an efficient technique because of its ability of linking ceramic/glassy materials to bioactive molecules. As a room temperature process, it does not denature biomolecules with harsh processing conditions. By sol-gel processing, thermally and electrochemically stable products in different forms with controllable surface area can be obtained. In addition, sol-gel derived microstructures offer high surface area and high sensitivity for biomolecule-analyte interactions [Bhatia and Brinker (2000), Satoh et al. (2006)].

In sol-gel encapsulation, because biomolecules are added to the system just before gelation, secondary formed silica colloids tend to aggregate around the biomolecules. Large number of hydrogen bonding groups on the surface of biomolecules result in extensive interaction with the silicate polymer which initally contains Si-OH-Si and Si-OH fragments. This means that biomolecules act as nucleus for condensation [Dave et al. (1994)]. The encapsulation mechanism can be seen in Figure 1.6.

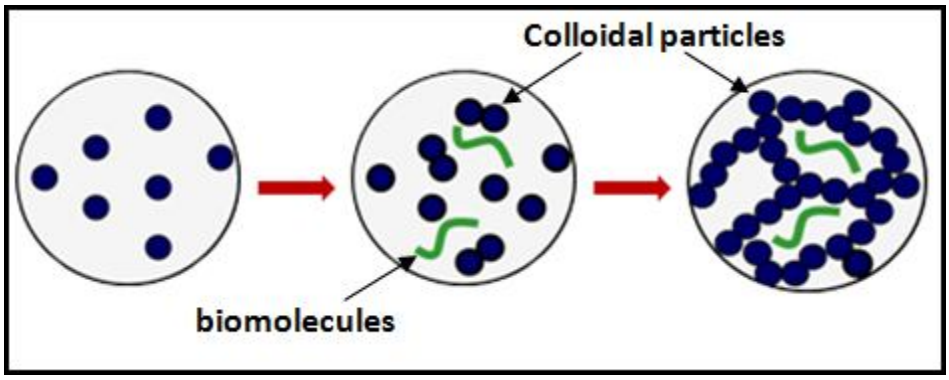

Figure 1.6 Schematic representation of encapsulation of biomolecules in sol-gel derived porous matrix

The encapsulation of biomolecules in sol-gel derived matrices has been recognized with the encapsulation of enzymes like horseradish peroxidase, globular proteins like myglobin, hemoglobin, cytochrome-c, etc. Sol-gel became apparent due to its recognizable processing conditions which are suitable for stabilizing fragile biomolecules. Especially enzymes showed better reactivity in sol-gel derived silica matrices then in their native solutions. In the study by Reetz et al., it was shown that activity of lipase enzyme could be enhanced up to 100 fold by using sol-gel encapsulation method [Reetz et al. (1995)].

By the time, encapsulation studies of many types of biomolecules like nucleic acids, proteins, enzymes, antibody-antigen complexes and whole cells became apparent. Antibodies or antibody antigen complexes were studied by Coradin and Livage [Coradin and Livage (2007)]. They produced a porous matrix derived by TMOS without using an alcohol to dissolve the precursor. They obtained an homogeneous mixture by sonication. They saw that antigens were not stable in the matrix, leaching out during the first washing step.

Whole cells like yeast cells have also been studied because of their potential of utilization in food industry, waste treatment and cell transplantation [Willaert and De Backer (1996), Livage et al. (2001)]. Pope et al. succeeded to protect in vivo pancreatic islet activity for three months after encapsulation [Pope et al. (1997)]. They also used an alkoxide based system. Different matrix materials like titania, alumina, silica have been used. For example, Yu and Ju studied titania thin films derived by vapor deposition for the encapsulation of horseradish peroxide enzyme [Yu and Ju (2002)]. But, as silica is so advantageous over other types of matrices, most of the encapsulation studies include silica or organically modified silica matrices. Silica is preferred over other inorganic materials due to its mechanical strength, chemical stability and non-toxicity. It does not swell in aqueous or organic solvents, not a food source for microorganisms and is biologically inert [Livage et al. (2001)].

1.2.3.1 Important sol-gel processing parameters for biomolecule encapsulation

Since all the biological interactions and stability are in direct relation with the sol-gel processing parameters and the conformational/chemical properties of the biomolecule, there are a variety of matrix types studied for different biomolecules. The gel formed by basic sol-gel route is a two phase system composed of an inorganic porous solid and the trapped solvent phase including the biomolecule. By studies of protein encapsulation, it became certain that pH, alcohol and the silica content are the key parameters for defining the biomolecule doped material properties. However, the processing parameters that are critical for the encapsulation of complex shaped biomolecules with dimensions in nanoscale such as nucleic acids are not clearly known [Avnir et al. (2005)]. It is harder to encapsulate small sized biomolecules because the silica cage cannot be formed effectively in sizes similar to those biomolecules.

1.2.3.1.1 Silica forming precursor type

The choice of the silica forming precursor is an important factor for the sol-gel processing of silica. Alkoxide based, alcohol free or colloidal silica derived matrices have been studied for encapsulation. For an alkoxide based system, TEOS and TMOS are the most common precursors. They are promising for the encapsulation of biomolecules like nucleic acids, enzymes, proteins and bacteria. The choice of the alkoxide mainly depends on the desired final matrix properties.

Alkoxide based precursors can be used for the encapsulation of bacteria. In a study obtained by Finnie et al. [Finnie et al. (2000)], TMOS precursor is used for the encapsulation of sulfate reducing bacteria. They found that the denaturation of the bio-organism was lower in TMOS derived matrix compared to TEOS derived matrix which may be due to the lower reactivity of TEOS.

The mechanism is the isolation of the bacteria in the pore cage with prevention of division of those cells due to space limitation. Thus, the reactivity of the silica forming precursor is an important parameter for size and scale of porosity that the bacteria become trapped. If the reactivity of the precursor is high, the encapsulation process would be faster and the space around the bacteria become limited. Nassif et al. concluded that they can become adaptable to those limited spaces, being culturable later by using *serratia marcences* [Nassif et al. (2003)]. Also, it is stated for many studies that mesoporous network obtained by this way is better for the retention of those cells.

1.2.3.1.2 Alcohol concentration

The alcohol amount is the primary factor in the protection of biomolecule conformation. Alcohol denaturation of the biomolecules may be reversible or irreversible depending on the type. Methanol denaturation, for example, is reversible for proteins (*cytochrome-c*) however, the effects of alcohols to the nucleic acid conformations are not certainly known. Besides, other conditions like the heat that is released while mixing water with alcohol also speed up the denaturation.

Since silica surfaces carry negative net charge at pH values higher than the point of zero charge (pH=2-3) and electrostatic interactions mainly depend on IEP (isoelectric point or point of zero charge) of the biomolecule, pH of the initial solution has critical importance [Livage et al. (2001)].

1.2.3.1.3 Pore size, size distribution and geometry

Another important factor for the encapsulation is the pore size of the matrices. Because biomolecules are highly sensitive to ionic constituents and the detrimental species or bacteria, pores should be small enough to prevent the passage of those inside with prevention of leakage of the biomolecule (other than bacteria). On the other hand, pores should be large enough for the passage of small nutritients. In most studies, biomolecules are added in their buffer solutions to the initial silica based sol, thus the sol-biomolecule interactions and then the interaction between them until the end of the process are critical for effective for encapsulation. Since the needed aqueous phases are also trapped in the pores for the encapsulation in those of porous matrices, pore sizes and distribution are the major parameters.

Shortly, the ideal encapsulation matrix material must act as an inert membrane for foreign molecules. In addition to these, when the ionic sensitivity of the conformational structure of the biomolecules come inside, pH becomes critical. Since pH is effective on biomolecule stability but also porosity, it becomes a more struggling work to succeed. It has been observed that materials, whose pore size just matches the protein molecular size, would have the highest activity and stability. Liu and Chen used hemoglobin which is a globular protein in 5.5 nm diameter and concluded that the effective pore size for encapsulation is around 5.8 nm [Liu and Chen (1999)]. Also for cell encapsulation, high porosity of silica gels favors water retention and nutrient diffusion allowing biochemical exchanges between trapped cells and the surrounding media [Livage et al. (2001)]. In addition to pore size, pore geometry is also important. The presence of small pores or bottlenecks even in big pores decreases diffusion efficiencies. If there are a large number of very small pores, higher amount of biomolecules are bound to the surface.

1.2.3.2 Different approaches for alcohol elimination

Using alcohol free routes or precursors with less detrimental alcohol contents or rotary evaporation for the elimination of by-product alcohols are possible. Organic based alcohol releasing precursors like poly-glyceryl silicates can be used to prevent detrimental effects of alcohols. Alkoxy based precursors mainly do not dissolve in water and necessitate catalysts for their reactivity.

However, these special precursors can overcome those problems. Polyol silicates liberate protective alcohols functioning also as drying control additives preventing the final stresses exerted on the monolith. The use of colloidal silica is also possible with alcohol free routes. They strengthen the fragile sodium silicate gels and avoid the excess osmotic pressure due to sodium.

Nassif et al. also studied those kinds of matrices and succeeded to protect the e-coli activity for a week [Nassif et al. (2003)]. Brennan et al. stated that in cases where proteins are destabilized by either low pH or high alcohol levels, encapsulation can result in loss of function. Greater the porosity, greater is the diffusion [Brennan and Jin (2002)].

1.2.4 DNA encapsulation in sol-gel derived silica matrices

For the conformation controlled bioactivity in solid materials, it is a challenging work to control the whole microstructure and biomolecule-material interactions. Thus, encapsulation of biomolecules with complex conformations such as DNA, RNA, cell membrane proteins and the protection of conformation sensitive activity necessitates a better defined material matrix and control of all properties in nanoscale. The uses of DNA encapsulation platforms either in powder or gelled form are critical for many biological and medical applications such as, environmental clean-up systems or bio-sensing devices [Satoh et al. (2006), Yamada et al. (2002), Yamada et al. (2008)]. DNA doped platforms can be used for mutation detection, gene discovery, DNA sequencing, chromatographic analysis systems, bio-sensing systems, etc. Thus, encapsulation of nucleic acids in bulk forms is a real challenge and depends mainly on the knowledge of structural properties and the interactions of the nucleic acid with the matrix.

DNA, which is the fundamental unit of life, has a very complex conformation whose activity is mainly determined by the protection of its conformation. It has a helix type covalent structure with a diameter of $20x10^{-8}$cm. The basic structure of DNA can be seen in Figure 1.7. The Watson-Crick base pairing is formed by combining A (adenine) to T (thymine) bases and G (guanine) to C (cytosine) bases.

If the sequence of nitrogeneous bases in one DNA strand is known, the other strand can always be reconstructed and this is the key recognizing event used in microarray technology. The directionality of the helix creates two important physical characteristics; one is the high flexibility of DNA and the second is its highly charged nature in water at room temperature. The sugar phosphate backbone is highly soluble in water and at physiological acidity levels phosphate groups are dissociated. DNA has an amphiphilic character that phosphate backbone is hydrophilic while the bases are hydrophobic.

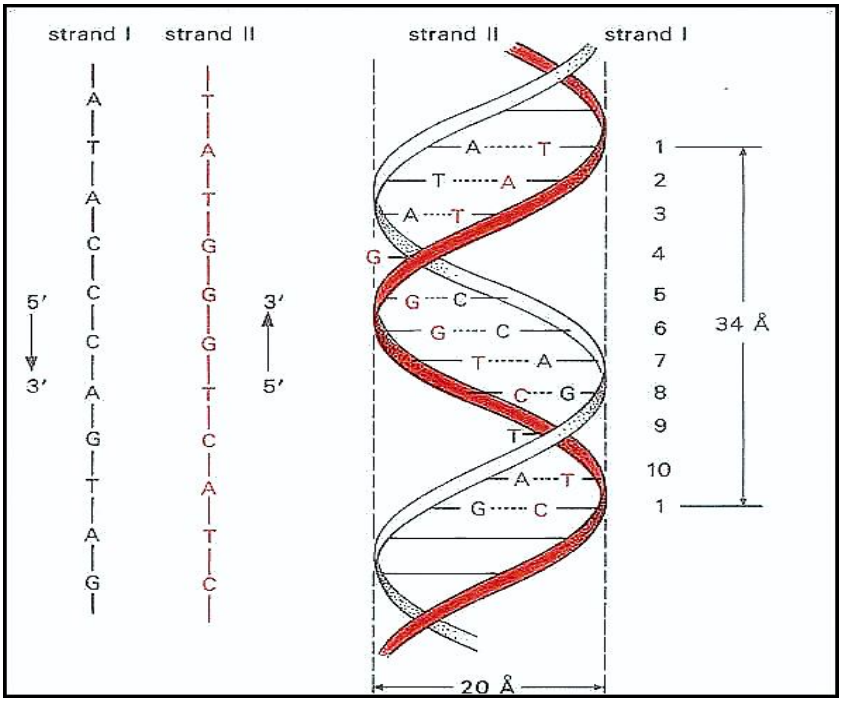

Figure 1.7 Schematics of DNA Structure showing the orientation of sugar-phosphate backbone strands and nitrogeneous bases (A, T, G, C)
(*Brown W., Willard Grant Press, 2nd Ed., 1976, Massachusetts, USA*)

Consequently, DNA needs to have its own helix structure to preserve its biological function in the organic media. Therefore the majority of DNA encapsulation must be to avoid irreversible conformational changes. When these are evaluated in terms of encapsulation in silica matrices, protection of the conformation and the presence of the aqueous phase are the primary factors to provide DNA stability in the matrix.

It is obvious that there is a limited knowledge about DNA encapsulation. One of the good examples that aim to determine the structure-DNA relations is studied by Pierre et. al. They studied the DNA encapsulation efficiency in different alkoxide and hybrid matrices derived by sol-gel technique. They stated that there becomes a complexation between the phosphate backbone of DNA and the silica matrix which prevents extraction of DNA from composites [Pierre et al. (2001)].

On the other hand, aptamers which are single stranded nucleic acids that are in vitro produced can be encapsulated in sol gel derived silica matrices. These kinds of products can be used in molecular recognition or as sensing elements. Rupcich et al. studied those microstructures derived by alcohol free routes and concluded that the overall degree of leaching is higher for DNA aptamers than in proteins [Rupcich et al. (2005)]. This effect is due to the electrostatic repulsion of DNA from the silica surface which could prevent the silica templating around DNA and thus result in relatively high mobility for the entrapped DNA.

In addition to alkoxide based and hybrid matrices, covalent modifications to alkoxide derived matrices is possible. APS (3-*amino prophyl triethoxy silane,* $SiC_3H_6NH_2(OC_2H_5)_3$) which is a common silane coupling agent that binds DNA molecules through its amine groups [Kato et al. (2005)]. Satoh et al examined those kinds of composites [Satoh et al. (2006)]. They used double stranded DNA molecules encapsulated in TEOS derived silica matrix. APS was used as a linking agent to improve DNA holding capacity. They mentioned that to protect the native conformation and functionality of DNA, it necessitates aqueous environment with mild pH and ionic strength. They concluded that an APS concentration of two percent is ideal for efficient DNA holding for that of matrix. By this way, they achieved higher than 90% of DNA incorporated in the structure.

As a consequence, from all those studies, it is obvious that there is a lot to investigate about the structural behavior of DNA in synthetic environments. These investigations would light the ways for many of critically important applications.

1.3 Objective of the thesis

DNA stabilization in/on solid materials has become a critical research area in the last decade. DNA immobilization matrices can be used in chromatographic analysis systems, diagnosis, biosensors, drug delivery and lab-on a chip/array technologies. Sol-gel derived encapsulation offers high and long term stability of DNA molecules which can make the utilization of such systems more common and may provide improvement in viability/of applications such biomedical devices and analysis systems. Through this fact, the general objective of this study was to encapsulate DNA molecules in sol-gel derived silica matrices without any denaturation.

The first specific aim of the study was to define sol-gel processing and material parameters that would provide protection of DNA and high encapsulation efficiency in porous silica matrices. Second specific focus of the study was about DNA helix-silica interactions during sol-gel encapsulation process.

In the first part, effect of sol-gel chemistry and incorporated DNA amount on gelation kinetics, chemical and microstructural properties of silica were introduced. DNA encapsulation efficiency could be compared by performing microstructural and chemical analyses. In the second part, the struggling work was about functionalizing the bulk DNA encapsulation matrices without disrupting the functionality of DNA molecules. For this purpose, the effects of aminopropyl triethoxy silane (APS) modification on the chemical and structural properties of hybrid silica (TEOS-APS) matrices were investigated. In order to determine the functional DNA amount that could be encapsulated and the DNA leach-out from silica, EtBr intercalation and DNA-leach out tests were applied.

CHAPTER 2

EXPERIMENTAL TECHNIQUES AND METHODS

2.1 Materials

The organic precursor used in making silica gels was tetraethylorthosilicate (TEOS, $Si(OC_2H_5)_4$, Sigma). In some sol-gel formulations it was used in combination with 3-amino-propyltriethoxysilane (APS, $SiC_3H_6NH_2(OC_2H_5)_3$, Sigma). The molecular structures of the organic precursors for silica are shown in Figure 2.1. The other chemicals used in thesis include ethanol (EtOH, C_2H_5OH, Merck), hydrochloric acid (HCl, 12M, Merck), ammonium hydroxide (NH_4OH, 15M, Merck) and Tris-EDTA buffer (TE buffer, 100x concentrate, 1M Tris-0.1M EDTA, pH≈8, Sigma, with designation number T9285). These chemicals were employed as solvent, catalyst and DNA stabilizing solution, respectively. TE buffer solution was diluted to 10x concentration with DI-water when necessary. In the formulations which were prepared to determine the DNA encapsulation efficiency, TE buffer was also used as the basic catalyst solution instead of ammonium hydroxide. All sol-gel precursors were reagent grade. Commercial grade acids/bases were diluted to desired concentrations with deionized (DI) water.

The encapsulation biomolecules used in this study were salmon sperm DNA which has a broad size distribution between 50-5000 base pairs (low molecular weight, double stranded, ≤5% protein impurity, Fluka, with designation number D31149). DNA was obtained in powder form. DI-water or TE buffer solutions were used to dissolve these powders prior to addition into sol-gel solutions. Another chemical that have been used was ethidium bromide (EtBr, $C_{21}H_{20}BrN_3$, Sigma, with designation number E7637). It has been employed as a DNA indicating fluorescent reagent in encapsulation efficiency studies.

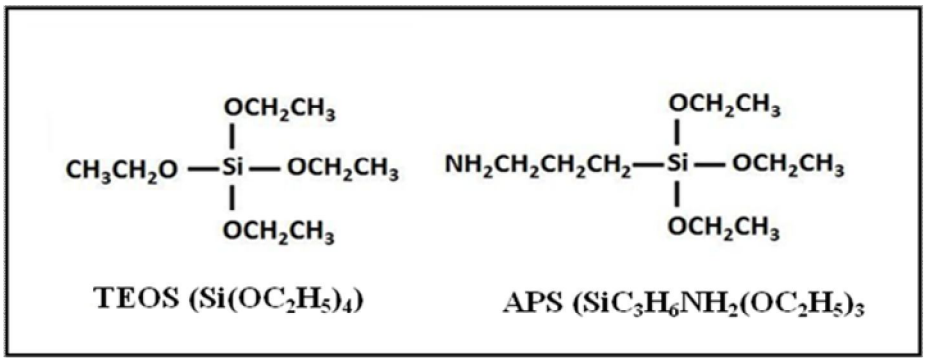

Figure 2.1 Molecular structure of organic silica precursors
(a) Tetraethylorthosilicate (TEOS, $Si(OC_2H_5)_4$) and
(b) 3-Aminopropyltriethoxysilane (APS,$SiC_3H_6NH_2(OC_2H_5)_3$)

2.2 Sol-gel process for encapsulation of DNA in silica

The standard sol-gel processing approach for DNA encapsulation is summarized with the flowchart shown in Figure 2.2. This approach simply relies on introducing DNA-containing aqueous solutions into the maturing TEOS derived silica sols before the completion of hydrolysis and condensation reactions. In a typical encapsulation process, silica precursor (TEOS) was dissolved in ethanol by mixing in 1:1 volume ratio in a borosilicate glass beaker at room temperature with a help of magnetic stirring bar. After obtaining a homogeneous solution by mixing for 10 min, aqueous catalyst solutions (0.1 M HCl, 0.1 M NH_4OH, 10x concentrate TE buffer solution or combination) were added drop-wise and the pH was monitored. After adding all catalyst solutions, the mixture was further stirred at 500 rpm for additional 5 min. At this point, while gelation reactions initiate and proceed, buffer solutions at different DNA concentrations were incorporated into the sol-gel mixture.

This was again achieved under continuous stirring by drop-wise addition. The pH was monitored throughout the addition process. The DNA added mixture was further stirred at 400 rpm for 5 min. The DNA-containing gels were then poured into polystyrene petri dishes or into test tubes for aging.

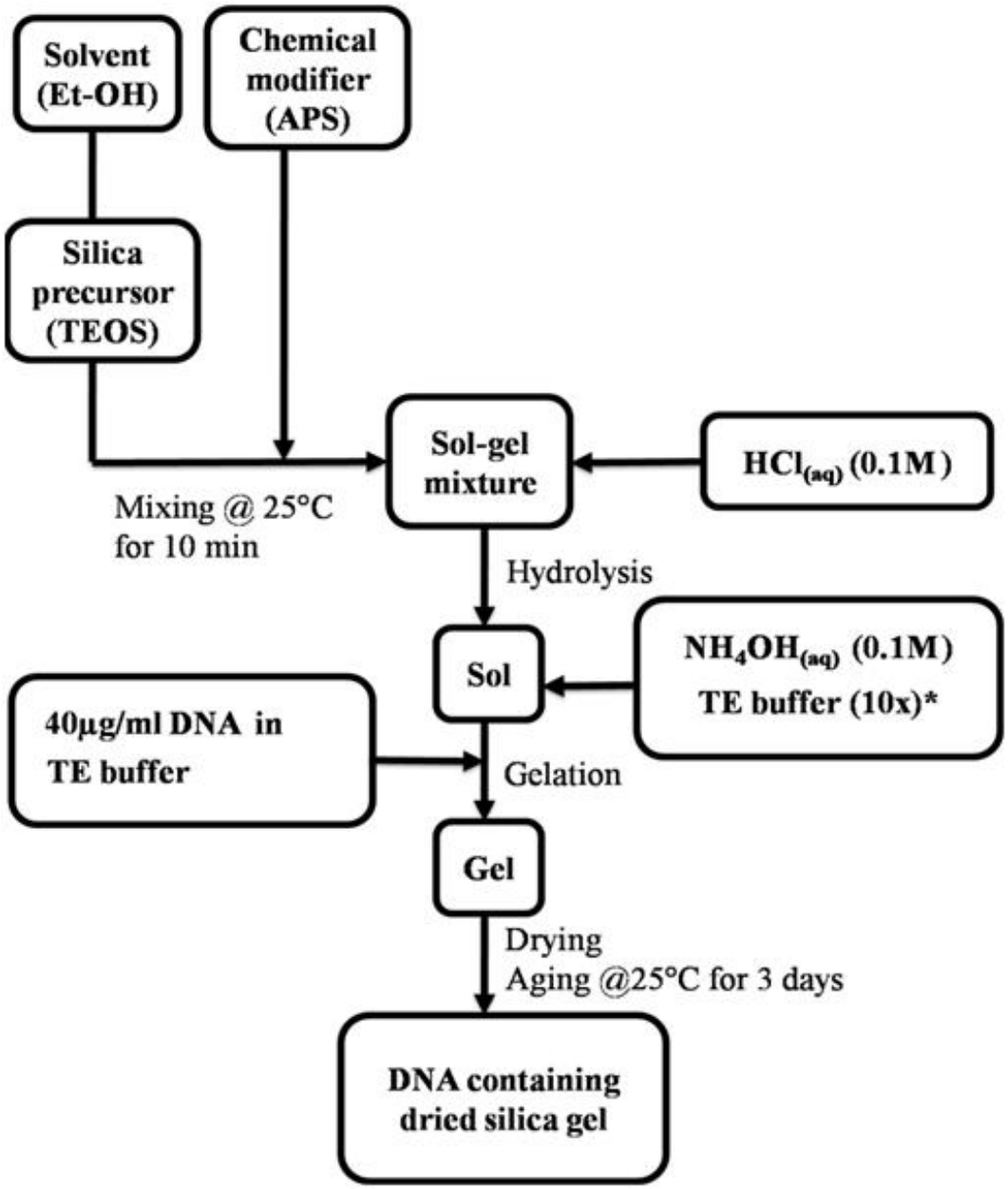

Figure 2.2 Standard sol-gel processing approach for pure and DNA containing silica gels
(*) Note that TE buffer (10x) was used as basic catalyst solution in DNA containing instead of $NH_4OH_{(aq)}$.

Aging was performed in ambient conditions, while the solution containers were kept sealed with Parafilm^{TM}. Typically, TEOS derived gels were transparent at this stage. The Parafilm covers were removed when the gels reached "syneresis" stage, where the liquid from hydrolysis/condensation by-products expel out from the gel network and accumulate on top of the semi-gelled products. This typically occurred within 3 days. The time period for complete gelation was different for different formulations, mainly controlled by the catalyst type, final pH and DNA concentration, as it will be presented in Chapter 3-4.

The gelation of the DNA-containing silica gels was monitored by visual examination. The complete gelation was considered as the state, when the gelling product reached an observed viscosity, preserving its shape and remaining intact without any distortion when tilted 45° angle. The gelling products in the test tubes practically enabled such examination. After gelation, bulk form gelled products were transparent or opaque, mostly controlled by the size and level of porosity. Later on, chunks of gels were ground into powders using an alumina mortar and pestle. These powder samples were then analyzed with different techniques for the investigation of microstructural and functional properties.

The detailed processing outlined above can be considered as the standard protocol for preparing DNA-doped silica gels. Pure silica gels in the same sol-gel formulations were also produced as reference to determine the chemical and structural differences upon DNA addition. Further manipulations in the formulations were achieved based on this approach. In one set, the modified gels were prepared with the addition of APS together with TEOS. Some turbidity was observed in APS-modified silica gels after mixing all the ingredients. In all cases, the conditions for gelling and drying were same as the standard protocol. Pure silica gels without any DNA addition were also prepared according to the same protocol.

2.3 Analytical techniques and characterization

One major objective of this study is to investigate the factors defining DNA encapsulation efficiency in silica matrices. The experimental work therefore focuses on establishing the processing parameters that may preserve the most possible activity and long term viability of DNA molecules without any functional loss in silica. In order to determine these processing parameters and their effects on structural and functional properties of the gel product, following investigations were performed with some analytical techniques and approaches. Performance-related "structural properties" and "sol-gel processing parameters" that have been introduced in this section.

Determination of gelation kinetics has been performed by visual observations and by simple solution chemistry (pH) measurements. Effects of catalysis mechanism, catalyst type and concentration and effect of hydrolysis media (water or buffer solution) on pure silica gelation were examined. Additionally, for DNA containing gels, the effect of incorporated DNA amount on gelation kinetics was also examined by varying the DNA concentration. Solid nuclear magnetic resonance spectroscopy (NMR) studies were also conducted to reveal the efficiency of silica network formation. Another structural examination for the gel was determination of scale and level of porosity. Those were achieved by gas adsorption (BET) technique. Determination of DNA encapsulation efficiency in silica gels has been performed by EtBr intercalation test.

2.3.1 Optical analyses (UV-vis Spectroscopy)

This technique is mainly used to determine the presence and concentration of organic entities and DNA in solutions and gels. In this thesis, it has been used for two purposes (i) in order to determine DNA state during sol-gel transformations and (ii) in EtBr intercalation tests to verify/determine the DNA amounts encapsulated in silica gel matrices for the evaluation of encapsulation efficiency.

A Varian Cary 100 Bio UV-visible spectrophotometer was employed in all optical analyses. Standard quartz cuvettes with a path length of 1cm and typically a sample volume of 3.5 ml were used.

2.3.1.1 UV-vis analyses for determination of DNA state during sol-gel transformation

Figure App.1.a in Appendix shows the UV-vis absorption of DNA containing buffer solutions in different concentrations. As the DNA concentration in the buffer solution increases, the absorbance increases and this relation is used to determine the concentration of DNA in solutions. Additionally, the linear relation between DNA concentration and characteristic UV absorbance at 260 nm was determined in Figure App.1b by using Origin Pro8 Program (Best fitting function). In this study, DNA concentration of the buffer solutions was fixed to 40 µg/ml TE for all DNA containing sample formulations. By the help of the characteristic UV-vis absorption behavior of DNA in this calibration study, the state of DNA at sol and gelled forms were examined.

First, DNA containing silica sol was analyzed by UV-vis spectroscopy. The gelation time was enough for measurement in the sol state. Later on, after the complete gelation and drying, the same DNA containing silica product was ground to powder form and suspended in DI-water at 10 mg/ml concentration.

All suspensions used in UV-vis absorption analyses were at this concentration. Measurements were done in 200-320 nm with a scan rate of 600 nm/min within 5 sec after suspending the samples in DI-water. DI-water has been used as the baseline correction solution in order to obtain the sole UV-vis absorption of DNA containing silica powders. Absorption of baseline solution was automatically eliminated by the spectrophotometer. In order to be able to comment on the state of DNA molecular structure in silica, the resultant data were compared to the absorption of UV-light by native DNA (pure) containing buffer solution (same concentration) with the DNA containing sol-gel formulations.

2.3.1.2 UV-vis analyses for evaluating DNA encapsulation efficiency (EtBr intercalation tests)

Ethidium bromide (EtBr, $C_{21}H_{20}BrN_3$) is a well known fluorescent reagent used as a DNA indicator. It can be used to indicate/evaluate the DNA presence and amount in aqueous solutions. EtBr molecules intercalate into DNA strands due to the aromatic structure given in Figure 2.3. High affinity of ethidium for DNA duplex is mainly based on electrostatic interactions with DNA helix and hydrogen bonding with phosphate groups in DNA backbone [Nafisi et al. (2007)]. Based on this information, in this study, EtBr molecules were used in some optical analyses (UV-vis) to determine the structure of DNA and towards elucidating its functional preservation in sol-gel derived silica matrices. EtBr intercalation test was also used as a quantitative tool to determine the relative amounts of encapsulated DNA.

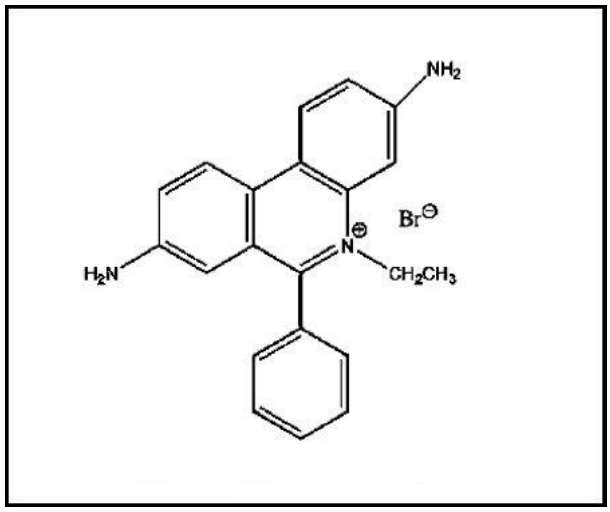

Figure 2.3 Molecular structure of ethidium bromide (EtBr)

EtBr intercalation testing procedure is illustrated by Figure 2.4. First, EtBr solution was prepared by dissolving EtBr powders in DI-water (10 μg/ml). Then, pure and DNA-containing silica powders were exposed to EtBr solution for 3 h. Later on, powders were separated from the EtBr solution by centrifugation and the supernatant solutions were analyzed by UV-vis spectroscopy. By comparing the changes in absorption maxima at 285 nm [Satoh et al. (2006)], which is specific to EtBr molecules, level of EtBr intercalation into DNA molecules in silica were determined. In Figure App.2a in Appendix, the change in UV-vis absorption intensity with respect to EtBr concentration in solution is shown. It is obvious that as the EtBr concentration in solution increases, absorption intensity increases. Pure EtBr solutions used in EtBr intercalation tests evaluated to determine the encapsulation efficiency in DNA containing silica samples were in 10μg/ml DI-water concentration. The level of EtBr intercalation by the samples were determined by comparing the absorbance intensity at 285 nm of pure EtBr solution (t=0) and EtBr solution after exposure to DNA containing silica powders for 3 h (supernatant solution, t=3 h). The intercalation of EtBr was also evident by visual examination; when EtBr molecules in the solution intercalate into DNA containing silica powders, the color of the supernatant solution changes.

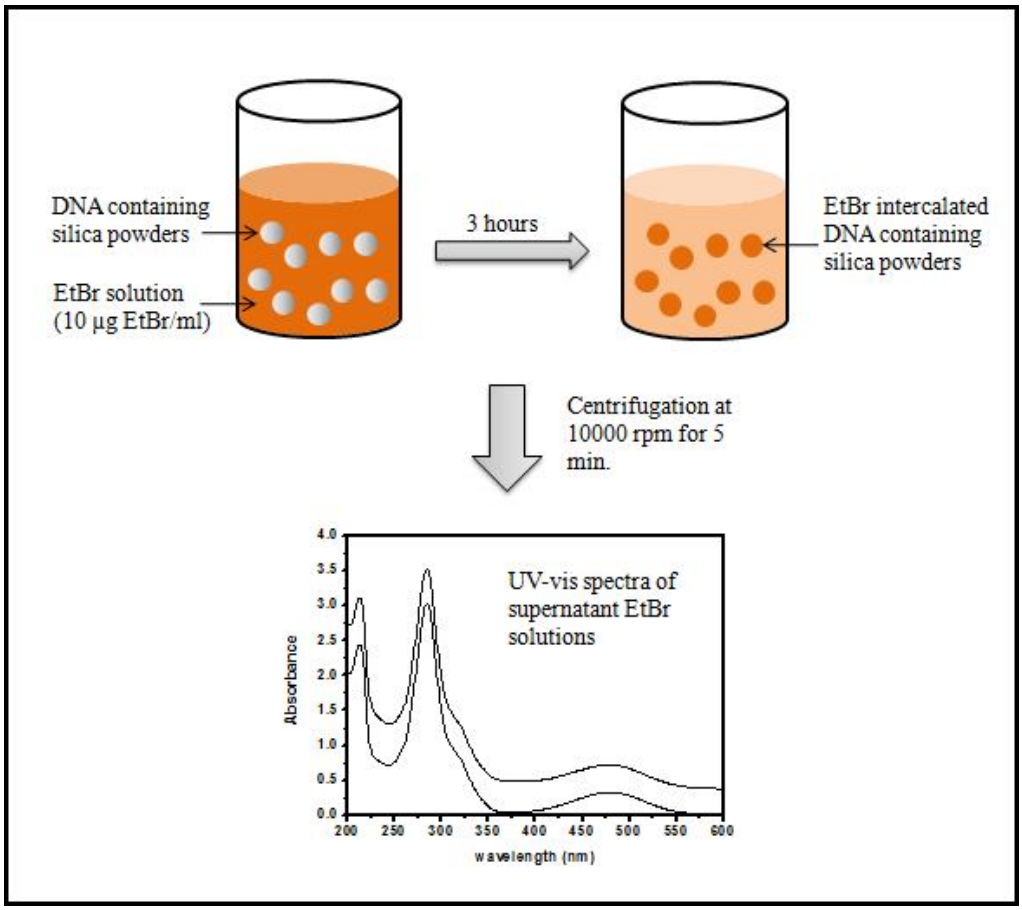

Figure 2.4 Schematic representations of UV-vis spectroscopy analyses for determination of DNA amount encapsulated in silica by EtBr absorption testing (The graphical data is incorporated only for presentation purpose).

2.3.1.3 UV-vis analyses for DNA leach out tests

Leach out tests were applied to DNA containing TEOS derived and hybrid (TEOS:APS) silica samples in order to investigate the DNA accessibility in silica matrices produced by encapsulation. A typical leach out testing procedure is shown in Figure 2.5. DNA containing silica powders in which DNA molecules were physisoprbed or encapsulated were exposed to pure TE buffer solutions for 2, 4 hours and 2 days. After the seperation of powdered samples from buffer solution by centrifugation, UV-vis absorption spectra of supernatant buffer solutions were taken and compared to the data for pure buffer solution.

The difference between them were related to the amount of extraction of species from silica and especially the presence of DNA molecules which leached into the buffer solution have been detected by the characteristic UV absorption band at 260 nm.

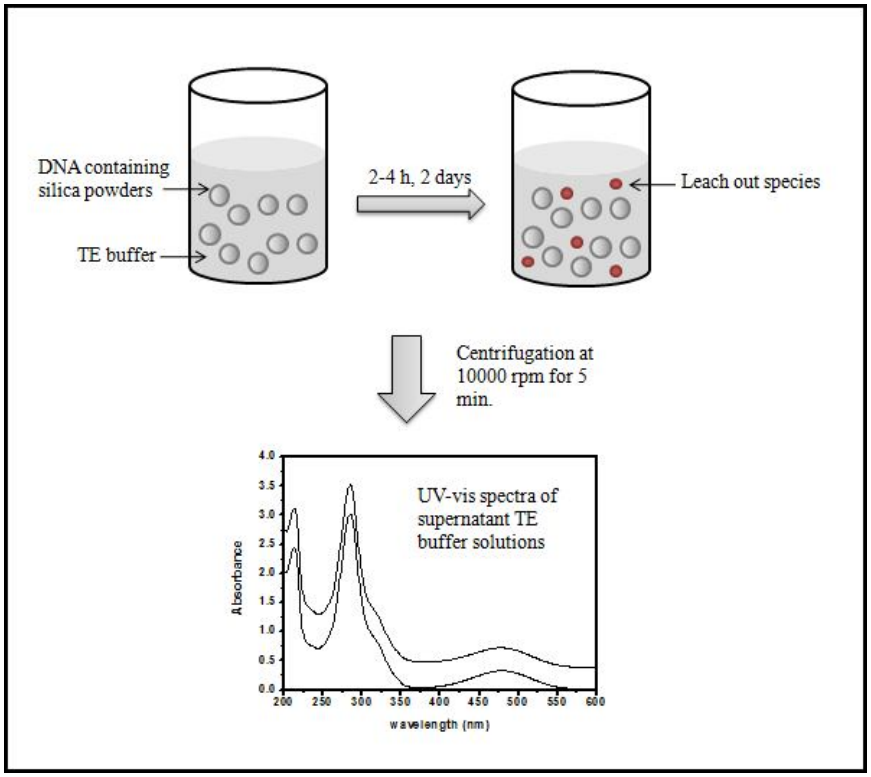

Figure 2.5 Schematic representations of UV-vis spectroscopy analyses for determination of DNA leach out testing (The graphical data is incorporated only for presentation purpose).

2.3.2 Molecular level structural analysis (^{29}Si NMR Spectroscopy)

^{29}Si NMR studies have been performed to determine the silica network formation extent in the sol-gel products. These analyses in turn provided a semi-quantitative measure of hydrolysis and condensation reaction rates depending on sol-gel derived silica chemistry. All of the NMR analyses have been performed in solid state. Bruker Superconducting FT-NMR Spectrometer (Avance^{TM} 300 MHz WB) was used. Relative percentages of resultant chemical species that correspond to the chemical bonding of silicon nuclei in sol-gel derived matrix were calculated by comparing the peak intensities.

The use of many other nuclei is possible for this analysis. This nuclei was also used to determine the chemically active sites on DNA phosphate backbone however, DNA amount doped to the initial formulations of sol-gel silica products were so small, that presence of DNA molecules in silica was not recognizable in ^{31}P NMR analyses.

2.3.3 Microstructural investigations: Pore size, size distribution and surface area determination by gas adsorption (BET Analyses)

The porous structure of pure and DNA-containing silica matrices were characterized by nitrogen gas adsorption according to Branuer, Emmett and Teller (BET) method. Quantachrome, Autosorb-1-C/Ms analyzer was used in gas adsorption studies. Powder samples used in analyses were approximately 0.2 g and all samples were degassed in high purity nitrogen gas at a temperature of 70°C for 16 hours before measurement.

BET uses the principle of measuring the amount of adsorbed gas volume which is adsorbed on the surface of the solid as a function of the pressure of this gas (P/P_0). The adsorption is performed in a temperature and pressure range just below the condensation point of the gas to liquid transition [Condon (2006)].

Average pore size of the materials were calculated by using BJH (Barrett, Joyner & Halenda) method based on the systems software which assumes a cylindrical pore shape with open ends. BJH/BET method of calculation allows to determine the distribution of the pores in the field of micro and mesoporosity. Porosity characteristics including average pore size, surface area and pore volume data of pure and DNA-containing silica matrices were compared to investigate porosity-DNA encapsulation efficiency relation.

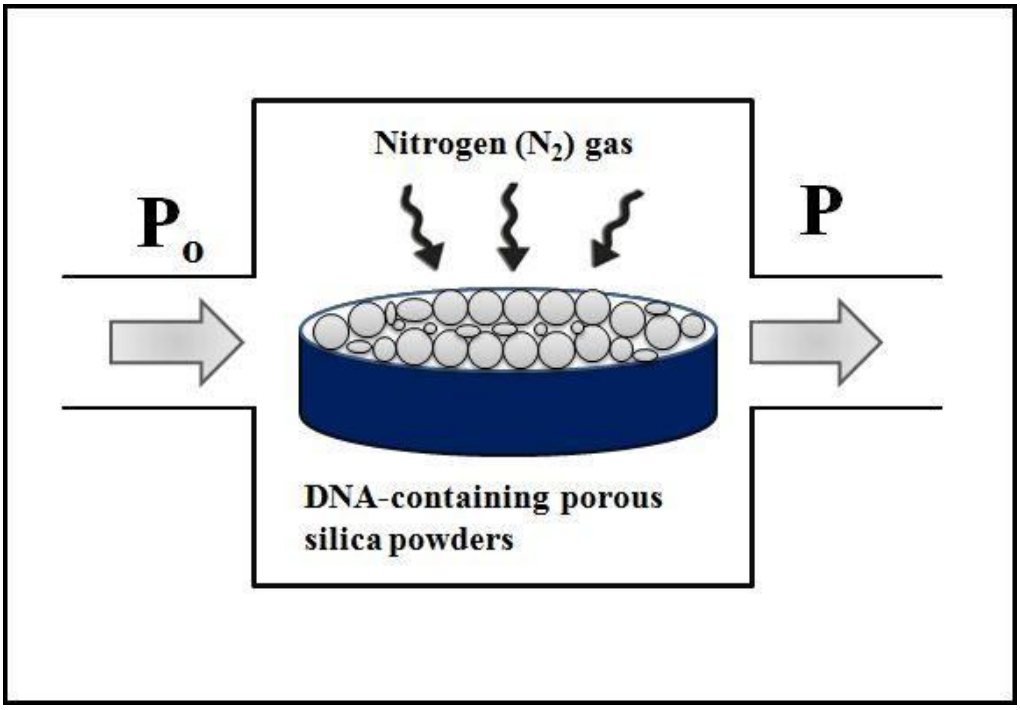

Figure 2.6 Schematics of gas adsorption measurements in BET technique.

CHAPTER 3

CHARACTERIZATION OF TEOS DERIVED PURE AND DNA-CONTAINING SILICA GELS

3.1 Gelation behavior of pure silica sols

3.1.1 Effect of catalysis mechanism on gelation

In order to study the effect of catalysts on gelation behavior, TEOS derived silica sol-gel formulations catalyzed at different conditions were prepared. The formulations of all pure silica sol-gel systems used in gelation studies are given in Table 3.1. Pure silica formulations given in Table 3.1 were prepared according to the processing route explained in Chapter 2 (section 2.2). Initially, for all formulations, TEOS precursor was dissolved in ethanol. For catalyzing hydrolysis and condensation reactions, 0.1M aqueous HCl solution and/or 0.1M aqueous NH_4OH solution were used. First sample (abbreviated as Ac) was acid-catalyzed and second sample (abbreviated as Bc) was base-catalyzed. A third sample (abbreviated as ABc) was prepared in a different way, where a two-step acid/base catalysis was achieved. In preparation of this specific sample, first acid catalyst ($HCl_{(aq)}$) was added to TEOS:EtOH mixture after mixing for 5 min and then base catalyst ($NH_4OH_{(aq)}$) was added.

The gelation time and final pH values for each silica sol are also given in Table 3.1. As mentioned in Chapter 2, the gelation time was estimated by visual examination, when the initial aqueous sol-gel reached a rigid form taking the shape of the container. The final solution pH values prior to initiation of gelation were in agreement with the catalysis mechanism.

The final pH values were 4.6 for the acid-catalyzed sample; 8.8 and 6.4 for the base, and two-step acid/base catalyzed samples, respectively. The fastest gelation was achieved for two-step acid/base catalyzed sample in which complete gelation was reached after 1 day.

The gelation times for acid- and base-catalyzed gels were 20 and 30 days, respectively. So, it is obvious that gelation behavior of silica sol was greatly controlled by the catalyst type and was pH dependent. This is very well established phenomena for metal alkoxide derived silica systems.

Table 3.1 Sol-gel derived silica formulations used in gelation studies, total gelation times and final pH of the solution after complete mixing prior to initiation of gelation.

Formulation (volume based, in ml) (TEOS : EtOH : $HCl_{(aq)}$: $NH_4OH_{(aq)}$: TE)	**Description**	**Final pH**	**Gelation (days)**	**Abbreviation**
25 : 25 : 2 : 0 : 0	acid-catalyzed pure silica	4.6	20	Ac
25 : 25 : 0 : 1 : 0	base-catalyzed pure silica	8.8	30	Bc
25 : 25 : 2 : 5 : 0	Acid/base catalyzed pure silica	6.4	1	ABc
25 : 25 : 2 : 5 : 1	Acid/base catalyzed pure silica with buffer	2.8	5	AbcT-1
25 : 25 : 2 : 5 : 3	Acid/base catalyzed pure silica with buffer	3	5	AbcT-2

The pH dependent gelation behavior of silica gels results from the difference in relative rates of hydrolysis and condensation reactions at different pH values [Brinker and Scherer (1985)]. This can be further clarified with the help of the graph previously given in Figure 1.5. As can be seen in Figure 1.5, pH value at around 2-3 (which is achieved during gelation of an acid-catalyzed gel) accelerates the hydrolysis reaction in an effective way. But, at around this pH value, condensation reaction is extremely slow. This leads to a very long gelation times for acid-catalyzed silica formulation (20 days for acid-catalyzed gel). Similar is valid for alkaline pH values in the range of pH=7-10. This time, condensation reaction is fast, but as the hydrolysis does not start efficiently, complete gelation occurs again at much longer times (30 days for base-catalyzed gel). For the two-step acid/base catalyzed gel on the other hand, an optimum overall gelation rate can be achieved. Initially, when the solution pH is reduced to a value at around 1-2 by addition of the HCl solution, hydrolysis reaction starts immediately and efficiently where condensation reaction still proceeds slowly. Upon subsequent addition of NH_4OH after a while the pH values will increase towards pH>7, where condensation is also favored. In this way, much faster gelation can be achieved (2 days).

This two-step catalysis approach may also promote some additional advantages towards DNA encapsulation beyond attaining faster gelation. Most biological molecules function in solutions that are at around physiological pH (7.4) values and they are sensitive to ionic conditions in solutions. During the two-step acid/base catalysis, the pH values of gels do not reach to extremities of very low or high pH, and gelation takes place at moderate pH values for the most of the time. The final pH value of 6.4 also implies the same; the gelation occurs and finalizes in an acceptable pH range potentially compatible for encapsulation of biomolecules. However, the gelation behavior of silica matrix in the presence of DNA can be different and one can expect variation due to presence of this additional component. Accordingly, the gelation behavior of silica was also investigated in the presence of DNA in coming sections.

3.1.2 Effect of buffer solution on gelation

One potential problem in introducing DNA to a silica sol is related to its aqueous stability. DNA is known to be stable in certain aqueous (buffer) solutions. DNA structure is highly sensitive to its hosting aqueous conditions and presence of ionic species. Additionally, the hydrolysis media can effectively change the hydrolysis and condensation reactions for TEOS, as mentioned earlier. So, before investigating the effect of DNA addition to gelation behavior of TEOS derived silica, the sole effect of addition of buffer solution to gelation behavior of pure silica was investigated.

The buffer solution used to stabilize DNA in this study was TE-buffer. Since acid/base catalysis gives the optimum gelation rate, the effect of buffer solution addition was investigated for the two-step processing route only. Formulations of buffer solution containing silica sols are also given in Table 3.1. The sample abbreviated as AbcT-1 contained relatively smaller amount of buffer solution compared to that of the sample AbcT-2. The gelation time and final pH values are also given in Table 3.1. TE-buffer addition decreases the final pH of the solution prior to gelation from 6.4 to 2.8, when the gelation of acid/base catalyzed pure silica (ABc) is compared with the data of ABcT-1. However, further increase in the buffer amount was not so much effective on pH. The pH was 3 when buffer addition was increased to three fold (by volume) as for ABcT-2. Additionally, gelation times for buffer solution containing gels show a dependence on their final pH. As the pH was decreased to acidic range by buffer addition, gelation time was increased to 5 days. From these data, it can be concluded that buffer solution acts as an acidic solution decreasing the pH of the solution. However, when the buffer amount was increased further, it was not so much effective on neither pH nor gelation time. This “acidic bleaching” effect of the buffer solution may be due to its highly ionic nature.

3.2 Gelation behavior of DNA-containing silica sols

As a followed up study on gelation, a set of silica gels with DNA addition with similar formulations to those of pure silica gels were prepared. The gelation behavior of these gels was compared to gelation of pure silica. Again, a two-step catalysis route was followed. In this set, DNA was introduced to the gelling sols by adding a DNA containing buffer solution to the sol-gel mixture just before excessive condensation (gelation) occurred. According to the previous observations on gelation behavior of pure silica gels, only two-step acid/base catalyzed systems reaching gelation in reasonable times were investigated. In Table 3.2, formulations, final pH and gelation times of DNA containing acid/base catalyzed gels are given. These formulations contained 0.5 mg DNA/ml and 2 mg DNA/ml buffer, respectively. Acid/base catalyzed pure silica data are also included in Table 3.2 for comparison.

Table 3.2 DNA containing sol-gel derived two-step acid/base catalyzed silica sol formulations used in gelation studies, total gelation times and final pH of the solution after complete mixing prior to initiation of gelation.

Sol-gel formulation (volume based) (TEOS : EtOH : $HCl_{(aq)}$: $NH_4OH_{(aq)}$: $DNA_{[TE]}$	**Description**	**Final pH**	**Gelation (days)**	**Abbreviation**
25 : 25 : 2 : 5 : 0	Acid/base catalyzed pure silica	6.4	1	ABc
50 : 50 : 2 : 8 : 5	Acid/base catalyzed /DNA incorporated silica	3.7	40	D-ABc-1
50 : 50 : 2 : 8 : 20	Acid/base catalyzed /DNA incorporated silica	7	1	D-ABc-2

According to the results shown in Table 3.2, the final solution pH values prior to gelation differed as a function of DNA amount. It was observed that the final pH of silica was decreased from 6.4 to 3.7 upon addition of DNA containing buffer solution (0.5 mg DNA/ml buffer, D-ABc-1). The gelation time also increased according to the pH drop reaching to 40 days for silica gels. For similar formulations without DNA, it was only 1 day. However, upon increasing DNA concentration in buffer further (i.e. when 2 mg DNA/ml buffer was employed, D-ABc-2), pH was increased to neutral range (pH=7).

Gelation times were again consistent with the final solution pH values. At this higher DNA loading, the gelation time was 1 day. These results showed that when the DNA concentration in the buffer solution is low, overall chemical nature of the buffer solution is the determining factor for gelation. In the case of low DNA-containing buffer addition, the final pH was acidic due to “acidic bleaching” effect of the buffer and resulted in longer gelation times. The gelation behavior of formulation was almost identical with buffer containing gels without DNA. On the other hand, when the DNA concentration in the buffer solution was increased further, gelation times became shorter due to the presence of DNA which leads to shorter gelation times. In that respect, DNA:buffer solution with substantial DNA amount can be considered as a “basic hydrolysis” media in sol-gel reactions.

3.3 Determination of DNA state during gelation

In order to address the effects of gelation (hydrolysis and condensation reactions) and hosting environment (silica gel) on the stability and state of DNA molecules, resultant DNA containing silica gels were examined by some analytical means. For this purpose, UV-vis absorption analyses of DNA-containing acid/base catalyzed silica was performed at two distinct stages of processing; prior to gelation *(in solution state)* and after achieving complete gelation *(gelled state)*. The characteristic absorption of the gels due to DNA presence in these conditions were then compared with the absorption behavior of a reference DNA solution.

The reference in this regard was absorption of a DNA solution with a pre-determined concentration. The amount of DNA in the reference solution was identical to the amount of DNA in the gel. Here, it is noteworthy to remember that pure silica is transparent to UV light (in the range of 200-400 nm). Therefore, any absorption event in DNA containing silica gels is specifically due to DNA itself.

The UV absorption spectra for DNA-containing gel in solution and gelled state are shown in Figure 3.1 together with that for the reference DNA solution. The native DNA in buffer solution (reference DNA solution) shows the characteristic absorption band of DNA at 260 nm. The DNA containing gel in solution (prior to gelation) shows a resemblance to DNA in native state in terms of absorption behavior. It also has an absorption band at 260 nm.

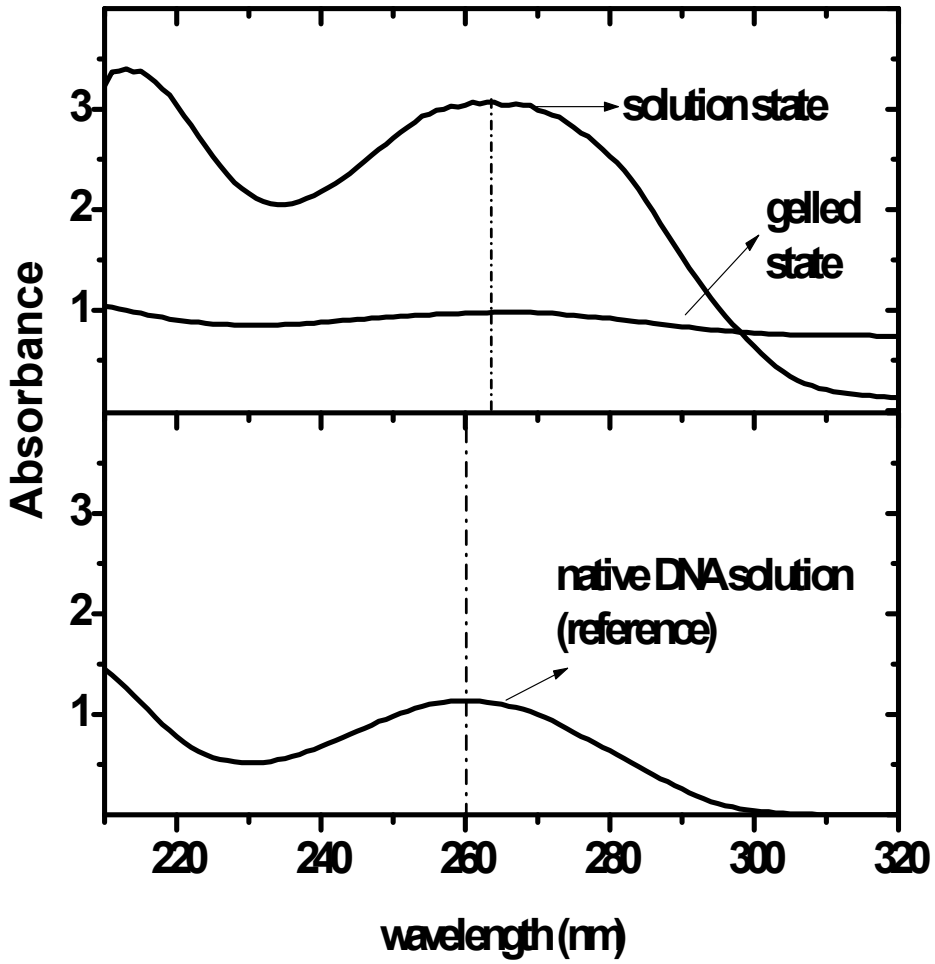

Figure 3.1 UV-vis absorption spectra of native DNA:buffer solution (reference) and DNA containing silica gel prior to gelation (*solution state*) and after complete gelation (*gelled state*) that DNA amount is identical in all cases.

The absorption spectrum of the gelled silica is distinctly different, with an obvious decrease in the intensity of 260 nm absorption (A_{260}) of DNA molecules. Taking all these into consideration, the absorption spectra given in Figure 3.1 can be interpreted in order to comment on what happens to the DNA molecules during gelation, i.e. during encapsulation in silica host. The DNA molecules in this study are salmon sperm DNA as pointed out earlier. The degree of contamination or change in the DNA state or change in the host can be determined qualitatively, by interpreting two factors; *i)* The change in absorption intensity due to DNA, *ii*) The change in characteristic wavelength of absorption maxima of DNA (A_{260}).

The resemblance in the absorption spectra of the native DNA in buffer solution and DNA containing silica gels prior to gelation suggest that the structure or state of DNA does not change that much in the early stages of the encapsulation process. As the absorbance of DNA mostly occurs due to the arrangement of bases in the strand, this also implies that the encapsulation does not lead to any denaturation or structural decomposition of the DNA. Thermal denaturation of DNA can be also a model phenomenon to support this conclusion. During melting of DNA when a double stranded DNA separates into single strands, the intensity of A_{260} increases [Lehninger (1981)]. This occurs due to the presence of free bases after the rupture of H-bonds between the base pairs that are available to interact with the incoming light. During denaturation of DNA by melting, while the absorption intensity changes; no other obvious change takes place in the absorption spectra.

But, one small distinction between the two spectra in Figure 3.1 is the position of absorption maxima, which differs when DNA is in buffer solution or silica gel. This is also pointed by the dashed vertical lines in the respective graph of Figure 3.1. The resultant absorbance data shows that, the absorption maxima of DNA shifts to higher wavelengths (2-3 nm) when it is present in the silica gel solution at its early stages of gelation.

The positional shifts in specific absorption wavelength may be due to a substitution into DNA molecules or due to changes in the local aqueous environment (solution). In this respect, the small wavelength shift seems to be related to the change in the local environment of DNA molecules, rather than a change in the DNA structure. So, during gelation and before the formation of solid encapsulation network, DNA molecules are free in the sol-gel solution similar to the state of DNA molecules in buffer solution. By further gelation, however, the obvious decrease in absorption intensity implies that the DNA molecules were in a new local environment and were not in native state as they were present in buffer or sol-gel solution. Once the semi-rigid/rigid silica network started to form, DNA molecules became entrapped or confined within the pores and absorption intensity decreased due to this new structural arrangement in new hosting environment.

3.4 Effect of DNA on silica network formation (^{29}Si NMR analyses)

As the sol-gel silica formation reactions and transition of a gel can affect the state of DNA during encapsulation, similar consideration is valid for the silica network. Presence of DNA may influence the chemical nature of the silica network and its formation mechanism. Such determination can be performed by NMR analyses provides molecular level information about the chemical nature of the silica (Si-O-Si network) during or after hydrolysis and condensation reactions. These analyses can also provide information about a possible molecular interaction between DNA and resulting silica matrix.

Towards characterization of the sol-gel derived silica, the analytical power of NMR relies on its ability in distinguishing the chemical (here silicon) species. In the case of ^{29}Si NMR, all the chemical species such as Si-OR, Si-OH, Si-O can be quantified for a silica product at any stage of the gelation process.

In the standard notation Q^n refers to the presence of n oxygen first neighbors around a silicon atom and the exponent "n" (from 0 to 4) represents the number of silicon neighbors. For example Q^2 refers to $Si(OSi)_2(OR)_2$ (R can be hydrogen or alkyl) and Q^3 refers to $Si(OSi)_3(OR)$. Similarly, Q^4 refers to $Si(OSi)_4$ where silica network formation is most complete without any unreacted hydrolysis and/or condensation species. The sum of Q^1, Q^2 and Q^3 species designates the amount of Si-OR groups, in other words, it shows the degree of incompletion in sol-gel reactions. On the other hand, a higher Q^4 value implies presence of totally inorganic Si-O-Si network.

For sol-gel derived silica ^{29}Si NMR chemical shifts, δ (in ppm) are assigned as Q^4 (-110 ppm), Q^3 (-100 ppm), Q^2 (-90 ppm) and Q^1 (-84 ppm) [Brennan (1999)]. One can evaluate the degree of silica network formation and identify of species comprising the sol-gel solution of any stage of gelation according to presence of these spectral assignments. Figure 3.2 shows the ^{29}Si NMR spectra of two silica gels of interest, i.e. pure silica and a DNA containing silica. It is noteworthy to point out that these silica matrices were obtained by acid/base catalysis with lowest possible acidity. This set was chosen rather a regular two-step acid/base catalyzed gel, as it provides a better model towards understanding the effect of DNA on the limiting reaction for gelation. As it was discussed earlier, in acidic catalysis conditions the condensation reactions are relatively slow (Figure 1.5).

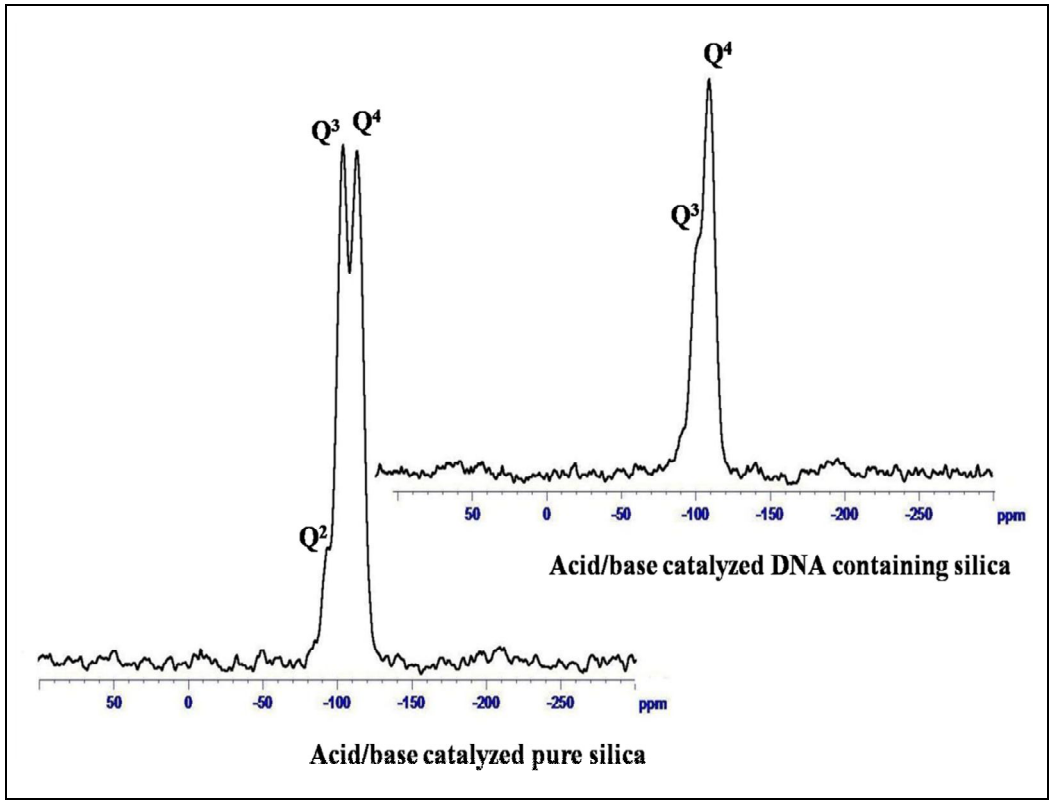

Figure 3.2 ^{29}Si NMR spectra for acid/base catalyzed pure and DNA containing silica gels.

Accompanying to Figure 3.2, Table 3.3 shows the relative percentages of the various species for acid-catalyzed pure silica and DNA-containing silica. These were calculated from the maximum intensity of the deconvoluated peaks of the respective species in the NMR spectra. Therefore it would be an accurate approach to compare the sum of relative percentages of Q^n species in which n=1, 2 and 3 in order to determine the level of structural incompletion in silica or Q^4 species to determine the completion level in each silica matrix. The data given in Table 3.3 shows that the condensation reaction in pure silica network was not complete. DNA addition promoted silica network formation. Q^4 species were increased from 44.5% to 62.5%. The same was apparent through the comparison of sum of Q^n species other than $Q^{4.}$ The level of incompletion ($\sum Q^n$ (n=1, 2, 3)) was decreased from 55.5% to 37.5% upon DNA addition.

The reason for such improved condensation in presence of DNA molecules may be due to the presence of active bonding sites on DNA sugar–phosphate backbone making the DNA helix behave as seeds in condensation of primary silica colloids. Besides, no additional Q^n species corresponding to DNA presence in the matrix were seen in NMR spectra. This shows that DNA molecules did not form a chemical interaction with the Si-O-Si network. The presence of DNA molecules enhanced the gelation and structural formation characteristics of the silica network.

Table 3.3 Relative Q^n percentages of pure and DNA containing acid/base catalyzed silica samples in order to investigate the effect of DNA on chemical nature of silica gel.

Description	**Q^4**	**Q^3**	**Q^2**	**Q^1**	**$\sum Q^n$ (n≠4)**
Acid-catalyzed pure silica	44.5	44.5	11	-	55.5
Acid/base catalyzed DNA containing silica	62.5	37.5	-	-	37.5

3.5 Effect of sol-gel chemistry on structural properties of DNA encapsulated silica gels

After showing the potential of sol-gel towards encapsulation of DNA, more detailed analytical characterization has been performed in order to understand structural properties of silica and also encapsulation efficiency of DNA. For this purpose, the effect of an important sol-gel processing parameter–catalyst concentration (pH)- on silica network formation efficiency and on the physical properties (porosity) of the resultant silica networks has been examined. Table 3.4 shows the DNA containing silica gel formulations employed. The formulations differ in terms of the catalyst concentrations used in the gelation process. All formulations had identical amount of DNA and also identical amounts of silica sol-gel precursors; TEOS and EtOH. In all cases, TEOS:EtOH was 1 (volume based). The main distinction between these formulations was the acidity. The HCl/total TE-buffer ratio was 0.2 and 0.05. Table 3.4 also shows the final pH and gelation times for sol-gel formulations. The final pH, prior to gelation, was around 7-8 for each case and the gelation was completed within the first hour for all formulations.

Table 3.4 Sol-gel formulations, final pH and gelation times of DNA containing silica gels catalyzed at different conditions in order to determine the catalyst concentration effect on encapsulation efficiency.

Sol –gel formulation (volume based) (TEOS : EtOH : $DNA_{[TE]}$	**HCl/TE (acidity)**	**Description**	**Final pH**	**Gelation (min)**
5: 5 : 4	0.2	Acid/base catalyzed	7.1	5
5: 5 : 4	0.05	Acid/base catalyzed (higher basicity)	7.8	60

Figure 3.3 shows the ^{29}Si NMR spectra of these acid/base catalyzed DNA-containing silica gels with HCl/TE ratios of 0.2 and 0.05. It is possible to investigate the effect of increased basicity (or decreased acidity) on silica network formation in the presence of DNA, since the total DNA amount contained in the silica network were identical in each sample. In Table 3.5, the relative percentages of Q^n species are given for acid/base catalyzed DNA containing silica gels with HCl/TE ratios of 0.2 and 0.05, respectively.

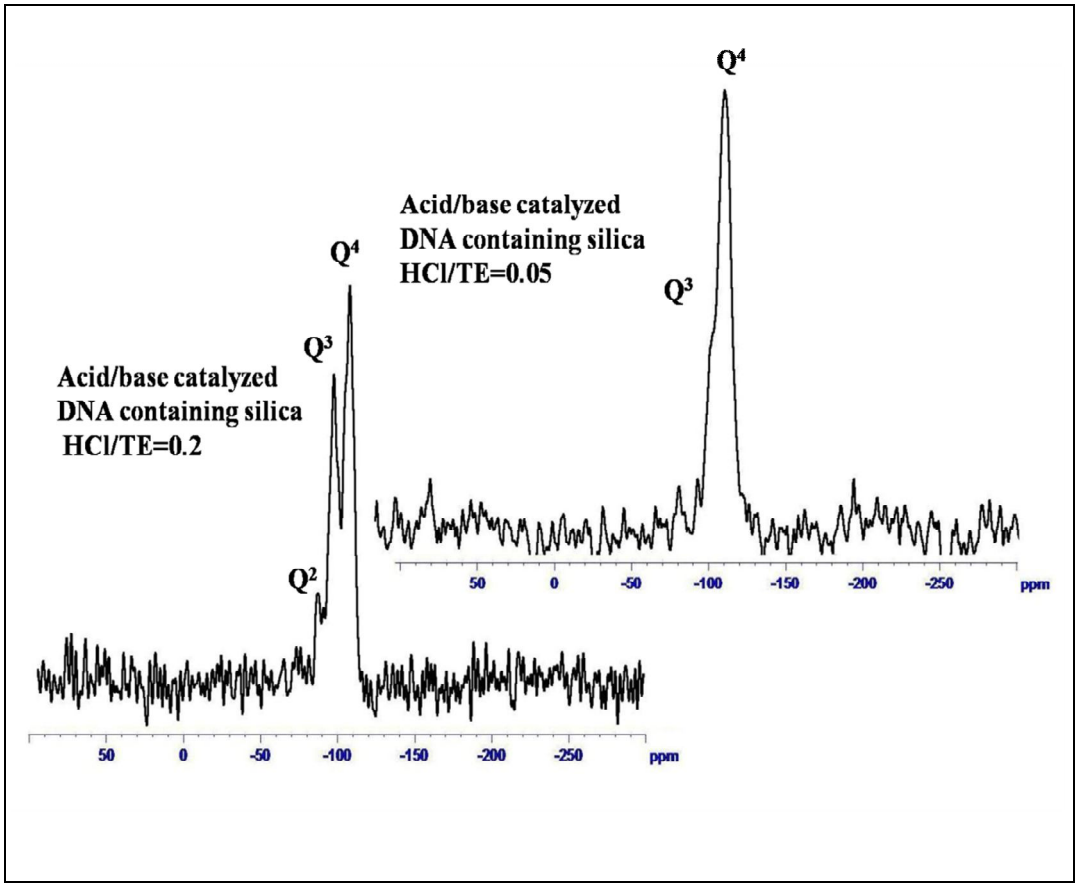

Figure 3.3 ^{29}Si NMR spectra for DNA containing acid/base catalyzed silica gels with HCl/TE=0.2 and 0.05.

Table 3.5 Relative Q^n percentages of DNA containing acid/base catalyzed silica samples in order to investigate the effect of catalyst concentration on chemical nature of silica.

Description	Q^4	Q^3	Q^2	$\sum Q^n$ ($n \neq 4$)
Acid/base catalyzed (HCl/TE=0.2)	47	39	14	53
Acid/base catalyzed (HCl/TE=0.05)	68	32	-	32

The results show that the relative amount of silica network formation was increased from 47% to 68% with increasing basicity. This may be due to the increased rate of condensation reaction increasing the Si-O-Si network formation degree with a decrease in Si-OH and/or Si-OR bonds. Silica network formation increases with accelerated condensation reactions due to increasing basicity in the presence of DNA molecules.

In addition to ^{29}Si NMR results, BET technique was used to investigate the physical structure (porosity characteristics) of these DNA containing silica matrices with different acidity ratios (0.2 and 0.05). In Figure 3.4-3.5, adsorption-desorption isotherms and pore size distributions calculated according to BJH method are shown. Table 3.6 also shows the size, scale and distribution of the porosity for each sample. As the DNA molecule has a fixed diameter of 2 nm with variable length, especially mesopore size (2 to 50 nm) and volume was taken into account.

It is obvious from BET results that acidity/basicity was highly effective on the properties of the final microstructure. Average mesopore size which was 5.6 ±1 nm for the acid/base catalyzed silica (with HCl/TE=0.2) increased to 17.5 ±1 nm with an increase in basicity (with HCl/TE=0.05). Mesopore volume (in P/P_0=0.1-0.6 range) of the matrix therefore should increase with increasing basicity due to the increase in the average pore size. However, there is a remarkable decrease in pore volume from 170 cm^3 to 31 cm^3 even though the pore size increases.

This can be explained by the increase in pore size in silica showing a macroporous nature which cannot be analyzed by the used BET gas adsorption equipment. Therefore it can be concluded that higher basicity increases the average pore size and changes scale of porosity “mesoporous” nature toward “macroporous”.

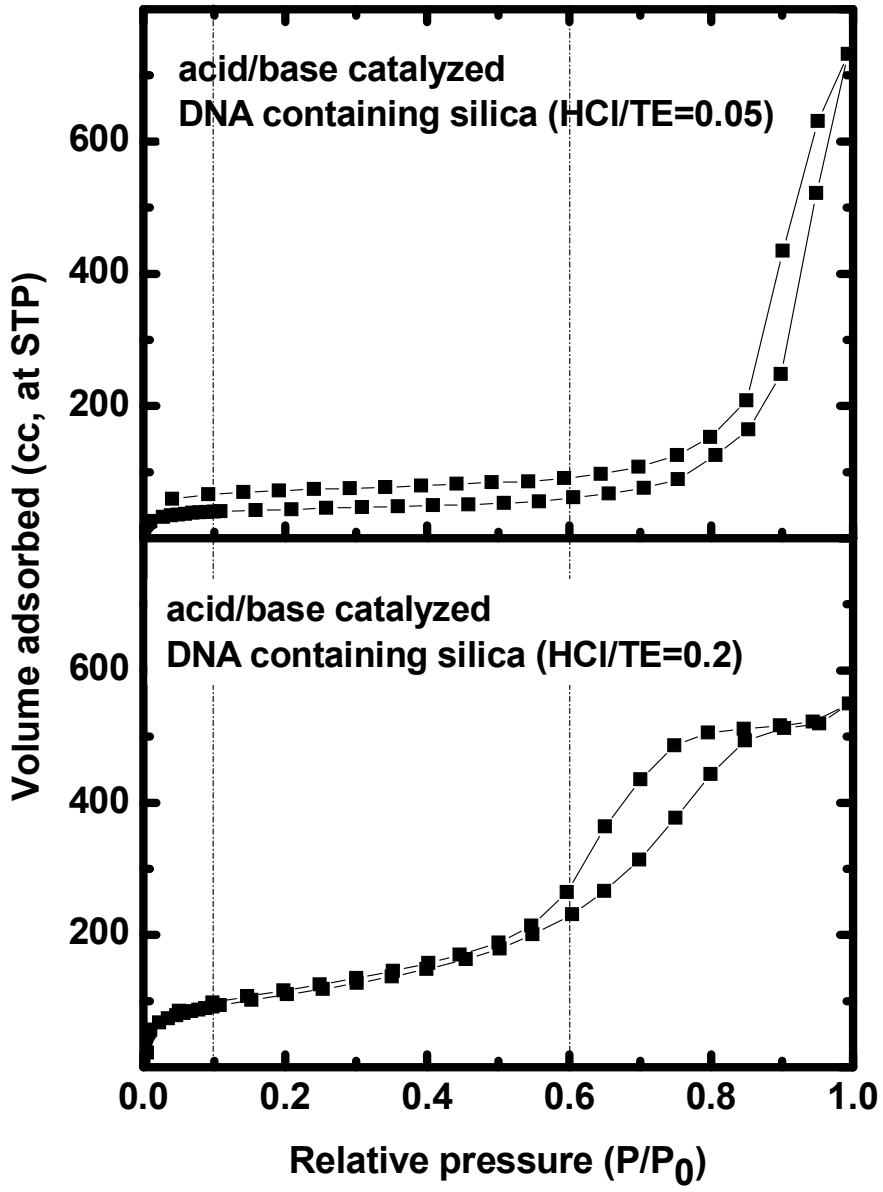

Figure 3.4 Adsorption-desorption isotherms of DNA containing acid/base catalyzed silica gels with HCl/TE=0.2 and 0.05.

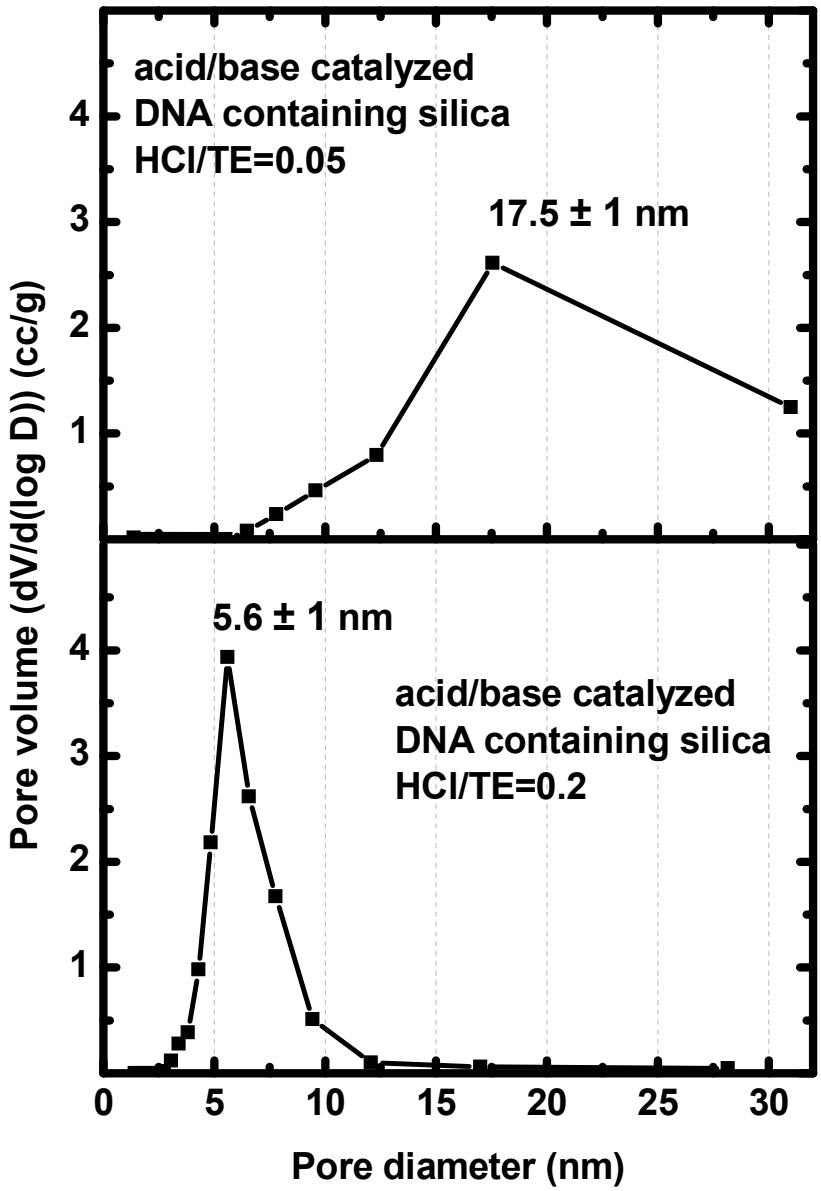

Figure 3.5 BJH pore size distribution for DNA containing acid/base catalyzed silica gels with HCl/TE=0.2 and 0.05.

Table 3.6 Porosity characteristics (average pore size, surface area and mesopore volume) of acid- and acid/base catalyzed silica gels (HCl/TE=0.2 and 0.05).

Sample description	Average pore size (nm)	Surface Area (m^2/g)	Mesopore volume (cc) (P/P_0 =0.1-0.6)	Pore size classification
Acid/base catalyzed (HCl/TE=0.2)	5.6±1	388	170	Mesoporous
Acid/base catalyzed (HCl/TE=0.05)	17.5±1	166	31	Macroporous

Overall, the catalyst concentrations apparently change the final chemical and microstructural properties of DNA containing silica matrices. The Si-O-Si network formation is promoted and the average mesopore size and pore volume increases with increased basicity. These are due to the accelerated condensation reaction which affects the gelation more dominantly.

3.6 Effect of DNA amount on structural properties of DNA encapsulated silica gels

It was obvious from the previous BET and NMR studies that higher basicity due to decreased HCl/TE buffer ratio promoted the silica network formation efficiency. It was seen that as the total TE buffer amount was increased, higher basicity promoted the degree of Si-O-Si network formation with an increase in average pore size. However, the effect of DNA on structural properties and encapsulation efficiency is not clearly known. In order to see the effect of DNA amount on structural properties of silica matrix, different sol formulations of varying amounts of DNA-TE buffer solutions were investigated. The sample formulations are given in Table 3.7. All formulations had identical amount of silica sol-gel precursors and the catalyst; TEOS, EtOH and HCl.

In all cases, TEOS: EtOH: HCl was 1:1:0.2 (volume based). DNA was added to the initial sol-gel formulation in TE buffer at a fixed concentration (40 µg/ml TE). The main distinction between sample formulations was the DNA-TE solution amount. The final pH was around 7.5-8.8 for each silica gel and the gelation time was approximately 2 min which consistent with the relatively high final pH values.

Table 3.7 Sol-gel formulations, final pH and gelation times of DNA containing acid/base catalyzed silica gels containing different amounts of DNA prepared to determine the effect of DNA on structural properties of silica and encapsulation efficiency.

Sol –gel formulation (volume based) (TEOS : EtOH : $DNA_{[TE]}$	HCl/TE (acidity)	DNA (µg)	Description	Final pH	Gelation (min)
5: 5 : 2	0.5	80	Acid/base catalyzed silica containing DNA (DAB-1)	7.5	2
5: 5 : 4	0.25	160	Acid/base catalyzed silica containing higher DNA (DAB-2)	7.6	2
5: 5 : 8	0.125	320	Acid/base catalyzed silica containing the highest DNA (DAB-3)	8.8	1

Figure 3.6 shows the ^{29}Si NMR spectra of the formulations employed containing 80, 160 and 320 μg DNA. It is possible to see the effect of increasing DNA amount on Si-O-Si network formation by comparing the amounts of Q^n species determined from ^{29}Si NMR spectra given in Figure 3.6. Table 3.8 shows the relative percentages of the Q^n species for acid/base catalyzed silica samples containing different amounts of DNA. Clearly, silica network formation proceeds more efficiently with an increase in DNA amount similar to previous findings. There was a remarkable increase in the amount of Q^4 species from 38 to 62.5% upon a two-fold increase in DNA amount (i.e. 160 μg) when it is compared to the silica containing 80 μg DNA. However, upon further increase, this effect was not apparent. For the silica containing 320 μg DNA, Q^4 species was 68%. This suggests that there might be a limit for DNA to be encapsulated in the silica matrices in an effective way.

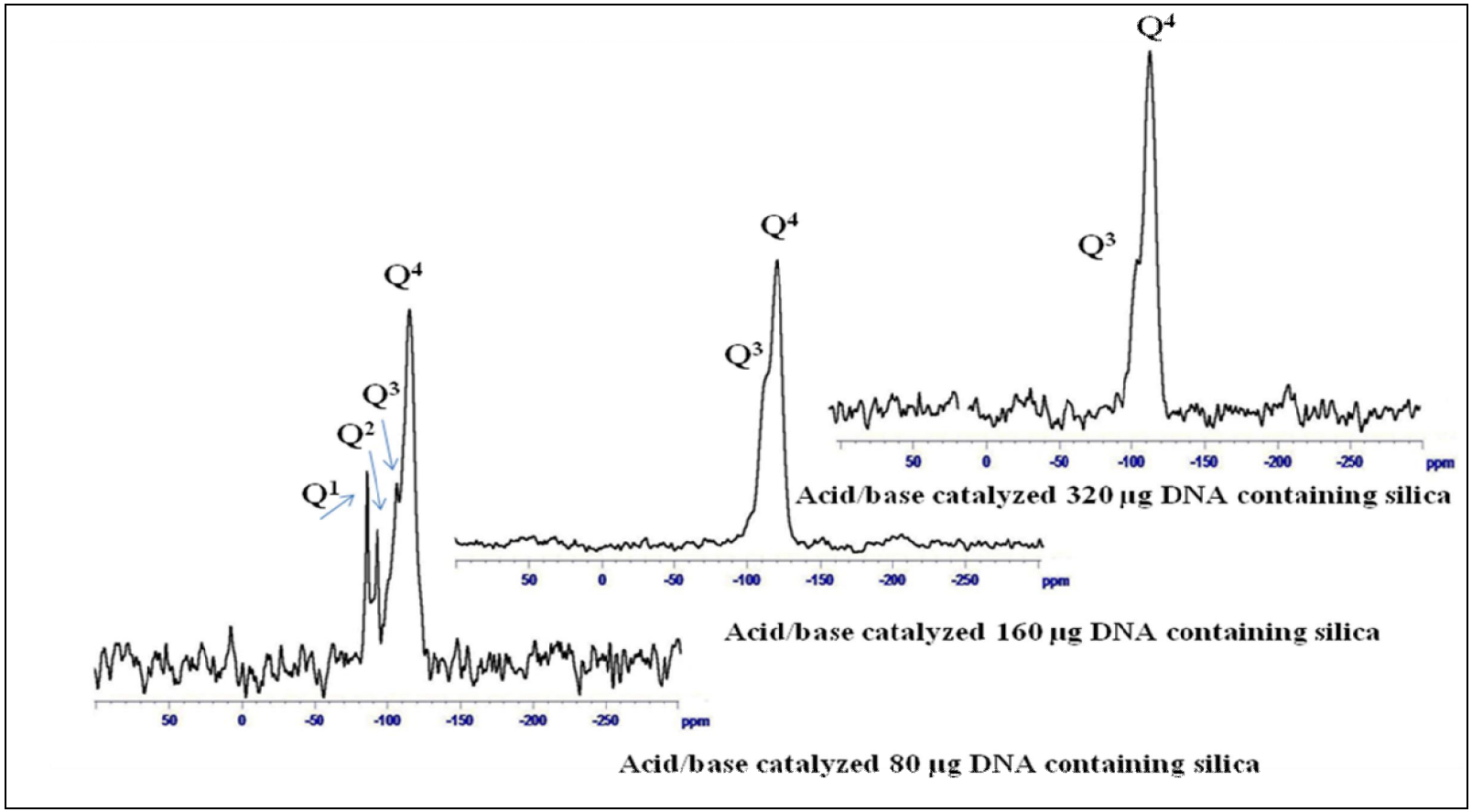

Figure 3.6 ^{29}Si NMR spectra of DNA containing acid/base catalyzed silica gels containing 80, 160 and 320 µg DNA .

Table 3.8 Relative Q^n percentages of DNA containing acid/base catalyzed silica gels containing 80, 160 and 320 µg DNA.

Description	**Q^4**	**Q^3**	**Q^2**	**Q^1**	**$\sum Q^n$ (n≠4)**
DNA doped acid/base catalyzed silica (80 µg DNA)	38	21	18	23	62
DNA doped acid/base catalyzed silica (160 µg DNA)	62.5	37.5	-	-	37.5
DNA doped acid/base catalyzed silica (320 µg DNA)	68	32	-	-	32

In addition to NMR results BET was also used to determine the effect of increased DNA amount on structural properties of the silica matrices. In Figure 3.7-3.8, adsorption-desorption isotherms and pore size distributions calculated according to BJH model are shown. Table 3.9 also shows the size, scale and distribution of the porosity for each DNA containing silica gel. As it was mentioned before, DNA molecules in this study have a diameter of approximately 2 nm with variable length, therefore, especially mesopore size (2 to 50 nm) and volume was taken into account. BET results show that there is a limiting value of DNA amount that can be effectively encapsulated in silica matrices. As the data given in Figures 3.7-3.8 and Table 3.9 show, average pore size of the matrices changes with DNA amount in the initial formulation. Average pore size decreased from 3.65±1 to 2.4±1 nm when DNA amount was increased from 80 to 160 µg. Mesopore volume data were also consistent with this decrease in mesopore size. However, within the limitations of the BET technique and BJH method, these differences are not that reliable. These two DNA containing gels can be considered similar in terms of physical properties (porosity) of the silica network. When DNA amount was increased to 320 µg, average mesopore size increased up to 10±1 nm. The adsorption-desorption isotherm also changes remarkably. The kick formed at high relative pressures in the isotherm data of silica with 320 µg DNA (given in Figure 3.7) indicates macroporous silica in the case of increased amount of DNA.

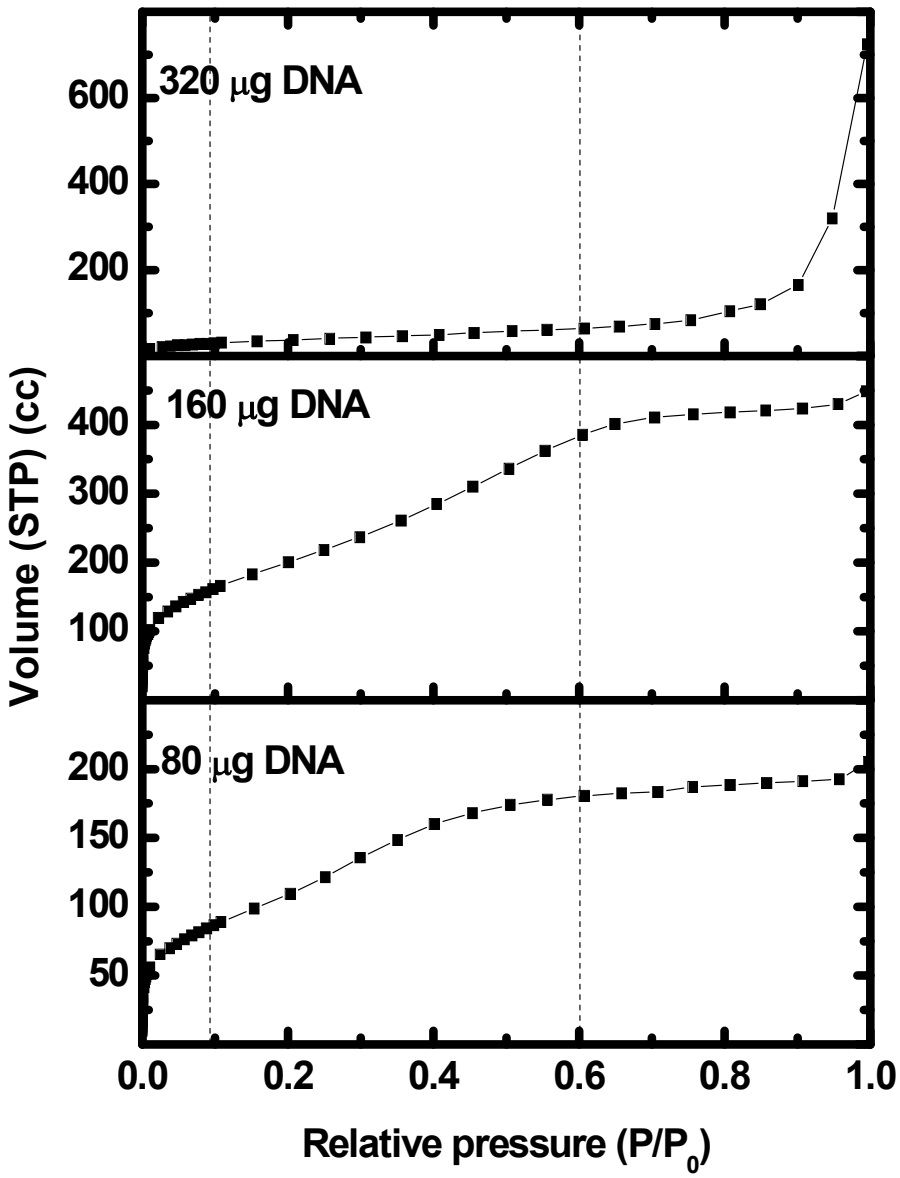

Figure 3.7 Adsorption-desorption isotherms of DNA containing acid/base catalyzed silica gels containing 80, 160 and 320 μg DNA.

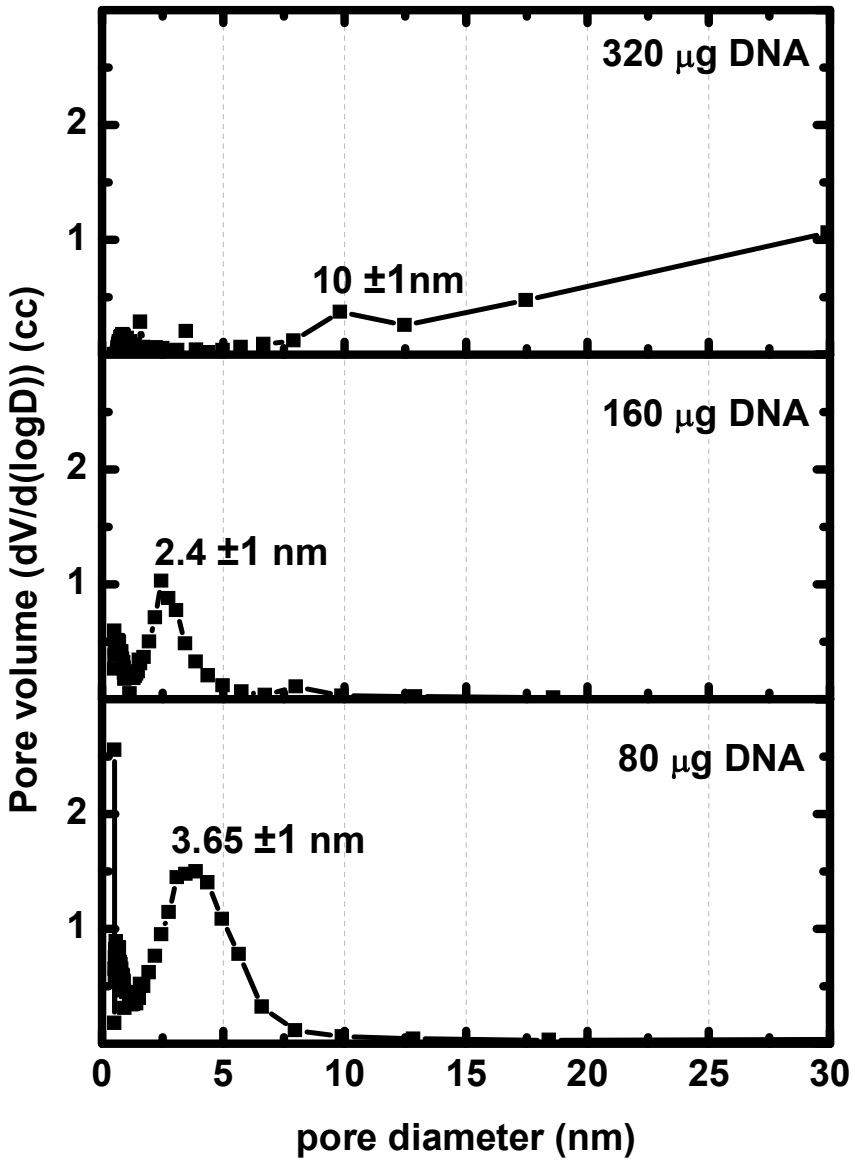

Figure 3.8 BJH pore size distribution for DNA containing acid/base catalyzed silica gels containing 80, 160 and 320 μg DNA.

Table 3.9 Porosity characteristics (average pore size, surface area and mesopore volume) of acid/base catalyzed silica gels containing 80, 160 and 320 μg DNA.

Sample description	Average pore size (nm)	Surface Area (m^2/g)	Mesopore volume (cc) (P/P_0 =0.1-0.6)	Pore size classification
Acid/base catalyzed (80 μg DNA)	3.65±1	750	219	Mesoporous
Acid/base catalyzed (160 μg DNA)	2.4±1	404	95	Mesoporous
Acid/base catalyzed (320 μg DNA)	10±1	137	33	Macroporous

It is obvious from both NMR and BET results that both average pore size and Si-O-Si network formation efficiency are strongly affected by the variation of DNA amount. These results indicate the importance of the pore size and whether it is comparable to that of the DNA molecule. Since the increased amount of DNA enabling a gelation chemistry leading to a structure with larger pores, the encapsulation which should enable presence of a single DNA molecule in porous silica matrices becomes difficult. This means that when the amount of DNA molecules increased in the gel formulation, bigger pore size causes a populated amount of DNA to be present in the larger pores. The encapsulation efficiency studies of coming sections should give some additional information about these factors and related interpretations.

3.7 Assessment of DNA encapsulation efficiency

In sections 3.5 and 3.6 structural properties of DNA containing sol-gel derived silica matrices were examined by NMR and BET analyses. It was concluded that two factors: i) catalyst concentration and ii) DNA amount are highly effective on structural properties of the resultant silica matrices. These parameters may also influence the DNA encapsulation efficiency. In order to see the effect of these factors on DNA encapsulation efficiency, EtBr absorption tests were employed according to the testing protocol explained in Chapter 2 (section 3.1.2). EtBr absorption test fundamentally gives insights about the DNA holding capacity of a solid host. Additionally, it gives indirect information about the state of encapsulated DNA molecules because EtBr is only absorbed by DNA molecules if they are present in the double stranded form. EtBr selectively intercalates into base pairs of the DNA helix.

In summary, in this test, the decrease in EtBr amount in aqueous solution, upon its specific and selective absorption on DNA containing sol-gel derived silica powders were measured. Due to its specific interaction with DNA, the variation in optical properties (UV-vis absorption) of EtBr solution can be correlated to the presence of functional DNA molecules in aqueous host [Satoh et al. (2006)]. Typically, there are two absorption peaks characteristic to presence of EtBr in a solution, one at 285 nm and another at 480 nm. Thus, by monitoring the change in intensity of these peaks, one can easily evaluate the variation in the EtBr amount in the solution. This occurs upon optical leaching due to time dependent attachment of free EtBr molecules onto/into DNA molecules. EtBr here simply acts as a fluorescent marker. The precise quantification of EtBr amount (absorbed by DNA containing silica powders) was accomplished according to the calibration measurements outlined in Appendix. One concern in these tests was of EtBr absorption on pure silica, which cannot be excluded due to a possible non-specific interaction. Therefore, pure silica powders were tested initially.

EtBr absorption to pure silica was analyzed by UV-vis spectroscopy by following the change in absorption spectra EtBr solution containing pure silica for 3 hours. UV-vis spectra and camera pictures of the EtBr solution in contact with pure silica are shown in Figure 3.9. Both the negligible color change and 3h spectra identical to initial one suggest that there was not any EtBr absorption to pure silica. So, non-specific absorption is not any and was excluded throughout the studies.

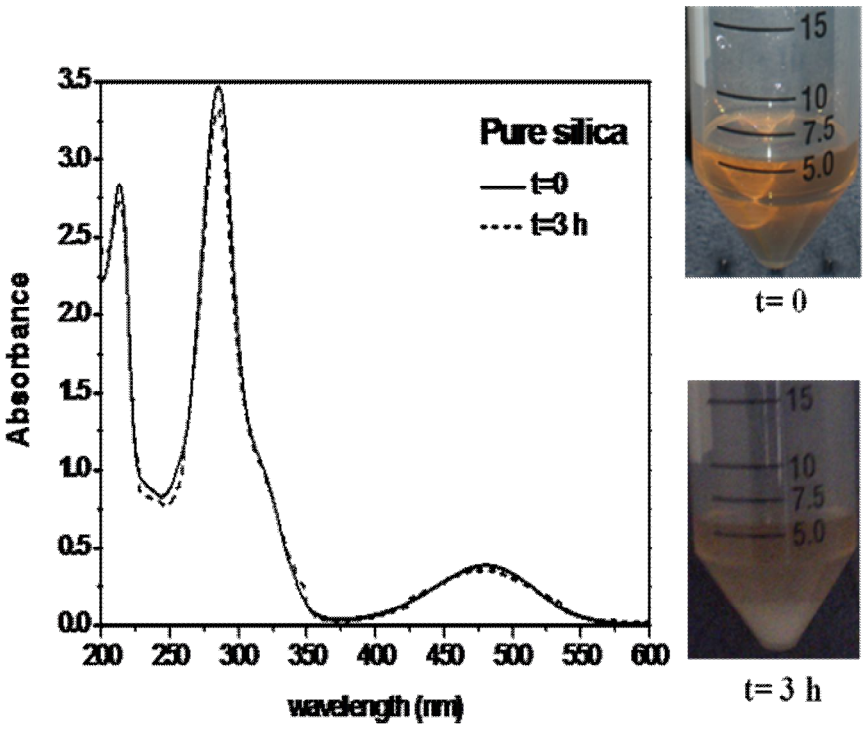

Figure 3.9 EtBr absorption spectra and pictures for reference EtBr solution (t=0) and EtBr solution exposed to silica powders for 3 h.

In order to investigate the effect of catalyst concentration on DNA encapsulation in silica, same formulations mentioned in Table 3.4 were examined by EtBr absorption test. Since all the samples had identical amounts of DNA, the sole effect of catalyst concentration (HCl/TE ratio, the acidity) on encapsulation efficiency could be examined.

In Figure 3.10, UV-vis absorption spectra of supernatant EtBr solutions of which were exposed to DNA containing silica powders for 3 h are shown. In order to evaluate the level of EtBr absorbance by DNA encapsulated in silica powders, absorption data of pure EtBr solutions (at 10 g/ml DI-water concentration) are also given for comparison purposes. This specific concentration corresponds to initial composition of the EtBr solution: i.e. before adding the DNA-containing silica powders. The absorbance (at 285 nm) difference between initial and the supernatant EtBr solutions after 3 h has been used to determine the absorbed EtBr amount as it was in previous literature [Satoh et al. (2006)]. The inset camera pictures in Figure 3.10 also provide a visual evidence/help showing how EtBr absorption test works.

As shown in these pictures, initially EtBr solution has orange color. After certain exposure to DNA containing silica gels (here after 3 h) as the EtBr molecules diffuse into the DNA molecules in the silica powder, the orange color fades out and becomes lighter. Similarly, the initial white color of the DNA containing silica powders slightly turns to orange. According to UV-vis measurements shown in Figure 3.10, the absorbed EtBr were found to be 56% for silica with HCl/TE=0.2 and 90% for HCl/TE=0.05. One should expect to see same or at least comparable DNA absorption efficiency on silica gels since the initial DNA contents were identical. But, EtBr absorption test results indicate a big difference in EtBr absorption trend (almost two-fold) for gels with same DNA amount. This implies that some structural differences are also crucial for DNA encapsulation.

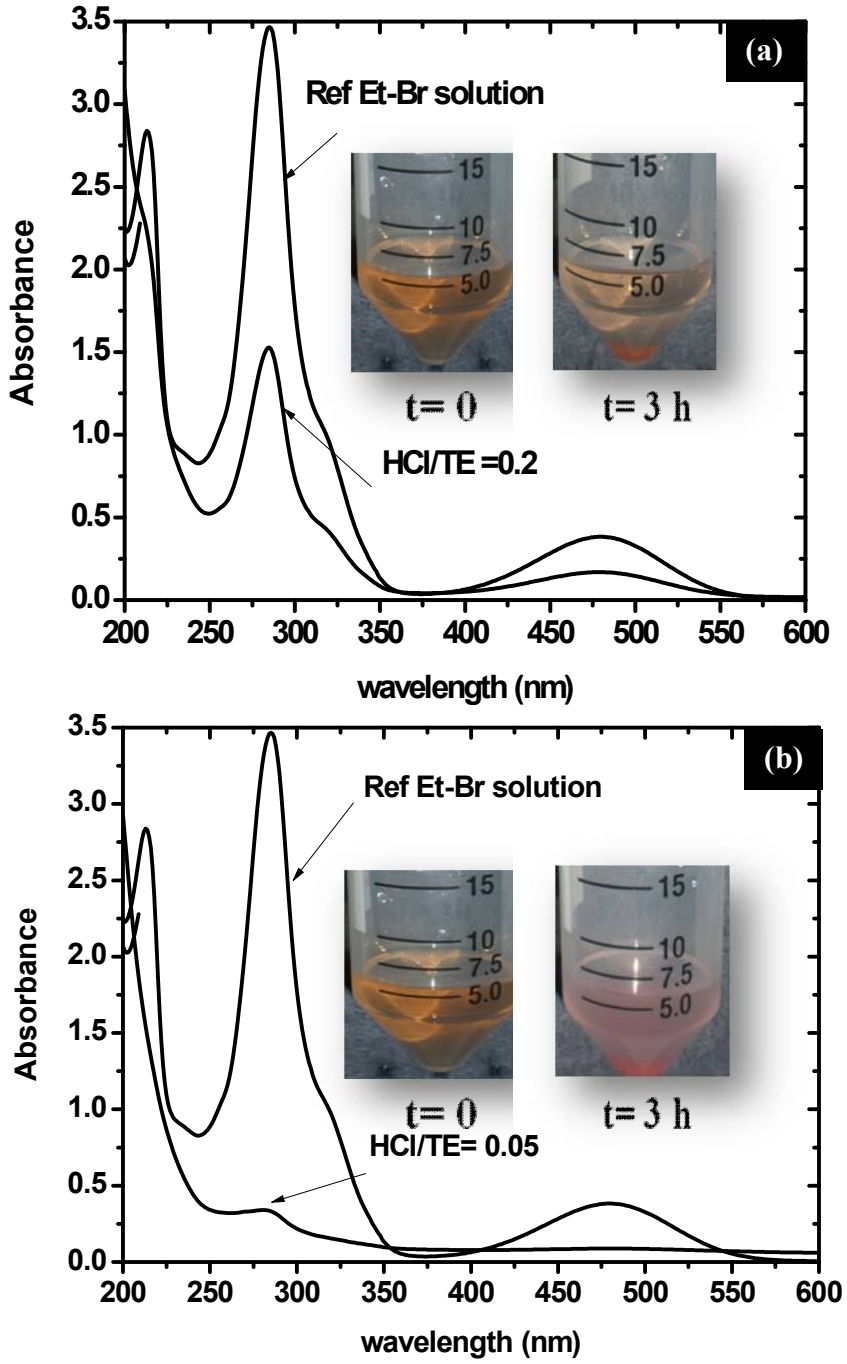

Figure 3.10 UV-vis absorption spectra for supernatant EtBr solutions exposed to silica gels containing identical amounts of DNA with different catalyst concentrations; **a)** HCl/TE=0.2 and **b)** HCl/TE=0.05. Note that each powdered silica was exposed to pure EtBr solution (at 10 µg/ml concentration) for 3 h. Insets are photos of EtBr solution at initial stage after interaction with silica powders for 3h.

As shown in previous structural analyses, the catalyst concentration mostly changes the silica network formation efficiency and the average pore size. It was found that the decrease in acidity (HCl/TE ratio) promotes a more complete Si-O-Si network formation with bigger pores (5.6 ±1 nm for silica with HCl/TE=0.2 and 17.5±1 nm for silica with HCl/TE=0.05). It can be said that DNA molecules were encapsulated in each silica matrix, but when the pore size reaches a critical limiting size, encapsulation becomes more inefficient. So, EtBr absorption test provide an analytical evidence for presence and encapsulation of DNA molecules in the sol-gel derived silica matrices. In fact, these examinations bring some additional valuable information on "encapsulation efficiency". This can be considered as a measure of preservation of the structural, chemical, functional state of DNA molecules in silica matrices, compared to their original native states when they are present free in a buffer solution.

Based on these, DNA-encapsulated gels, containing various amounts of DNA can be used as model structures to further investigate this postulation. Thus, the effect of DNA amount on encapsulation efficiency was also investigated by EtBr absorption test. The sample formulations were the same those employed in section 3.6 (Table 3.7) with varying DNA amount. Figure 3.11 shows the UV-vis absorption spectra of supernatant EtBr solutions which were exposed to silica powders containing different amounts of DNA for 3 h. The difference in UV absorption spectra after interaction with DNA containing silica powders can be clearly seen for each sample. In all cases, a comparable decrease in the A_{285} absorption peak has been observed, suggesting an uptake of EtBr molecules by the DNA containing gels. Accordingly, absorbed EtBr amounts for the silica samples containing different amounts of DNA were calculated. EtBr amount absorbed by the silica containing 80 µg and 160 µg DNA were 36% and 70%, respectively. This means that EtBr intercalation increased with increasing the initially incorporated DNA. Up to some fraction, DNA molecules in each silica were functional after encapsulation. As shown in the previous structural analyses, the average pore sizes for these gels were 3.65±1 nm and 2.4 ±1 nm, respectively.

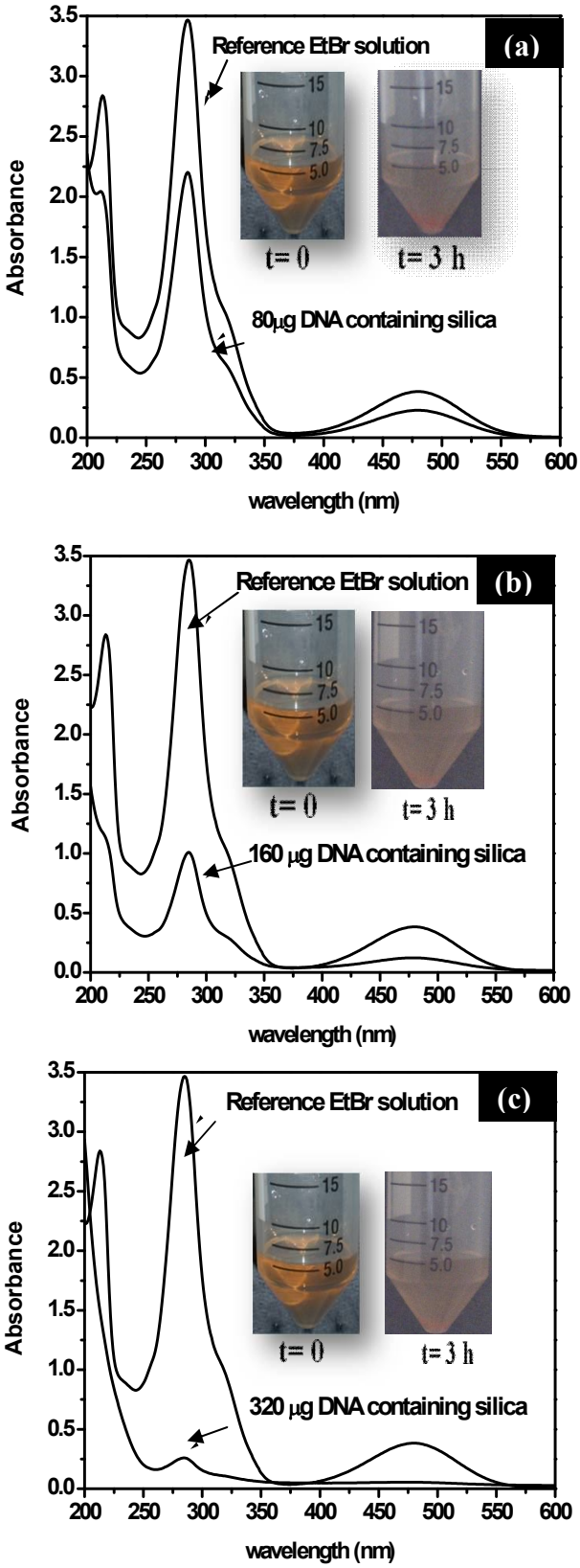

Figure 3.11 UV-vis absorption spectra for supernatant EtBr solutions exposed to silica gels containing different amounts of DNA for 3 h; a) 80 μg b)160 μg and c)320 μg DNA. Insets are photos of EtBr solution at initial stage after interaction with silica powders for 3h.

With 80 μg DNA incorporation, the intercalation was 36% into the 3.6 ±1 nm sized pores. It was expected that a linear relation between the DNA amount and EtBr absorbance could exist. But EtBr intercalation increased up to 70 % with 160 μg DNA addition. On the other hand, for the highest amount of DNA incorporated gel formulation – 320 μg DNA- the EtBr uptake remarkably increased to 92 %. The average pore size for this high DNA containing gels was previously measured as 10±1 nm. This suggests that the average pore size becomes a limiting factor for DNA holding capacity. DNA is highly effective on the sol-gel chemistry and thus the average pore size. As the amount of DNA increases, the silica microstructure becomes more porous and more amount of DNA molecules become encapsulated. However, since the DNA strands are not equal in length, average pore size- or the size match between the DNA strand and the pore- becomes more critical on encapsulation efficiency.

There are some critical conclusions obtained by EtBr absorption test. First of all, it works as a semi-quantitative analytical tool to assess presence and encapsulation of DNA in silica matrices. Additionally, it also shows that the encapsulated DNA molecules are preserved in their native state. As EtBr absorption requires double stranded DNA molecules, it looks like the chemical and the structural changes during formation of silica hydrolysis and condensation reactions are not detrimental on form and state of the DNA molecules. Finally, EtBr absorption test also shows that there may be a direct absolute correlation between the amount of DNA initially present in sol formulation and DNA that had been encapsulated. But, the encapsulation efficiency (or DNA holding capacity) for the sol-gel derived silica strongly depends on the scale and the size of the porosity attained. An efficient encapsulation requires a size match between the average pore size and length of the DNA molecules. The DNA used in this thesis was commercially supplied salmon sperm DNA mostly comprised of variable lengths of 50-5000 base pairs.

There should be also some DNA fragments with a length shorter than 50 base pairs at some small fraction. The spacing between neighboring base pairs in a DNA helix is around 0.35 nm [Lehninger (1981)]. The typical pore size for the sol-gel derived silica matrices are on the order for 5-20 nm. This means these gels can accommodate the DNA fragments with 15-60 base pairs.

The pore size dependent encapsulation efficiency and its correlation with the size of DNA fragments can be explained with the schematic in Figure 3.12 and Figure 3.13. At such "pore size match" requirement, if the average pore size is big; then all the DNA fragments with a size similar to the average pore size or smaller will be effectively encapsulated. This will correspond to encapsulation of DNA fragments of relatively broader size distribution; the upper limit being size of the pore. If the pore size is too small, then there would be much more limited amount of DNA fragments with appropriate sizes matching with the pore size.

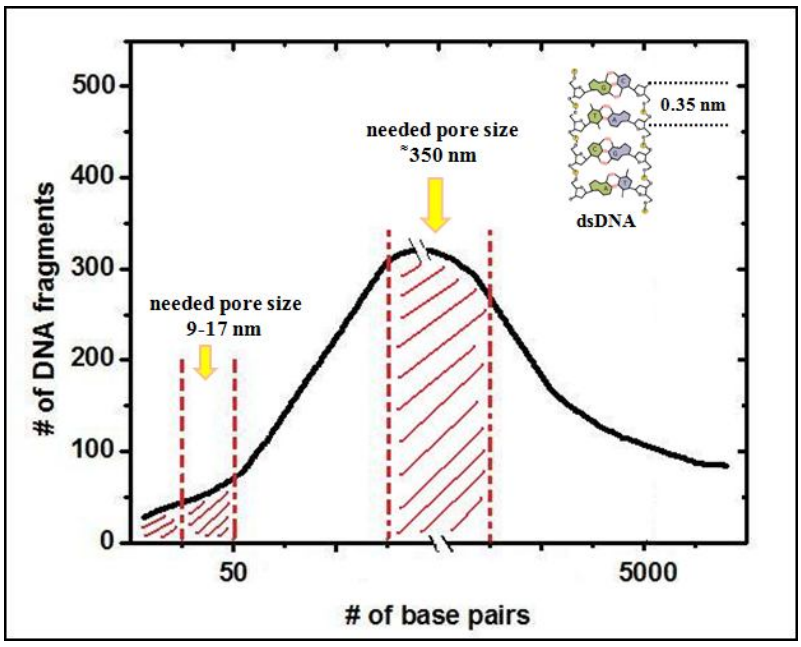

Figure 3. 12 Schematics of size distribution of commercially supplied salmon sperm DNA molecules.

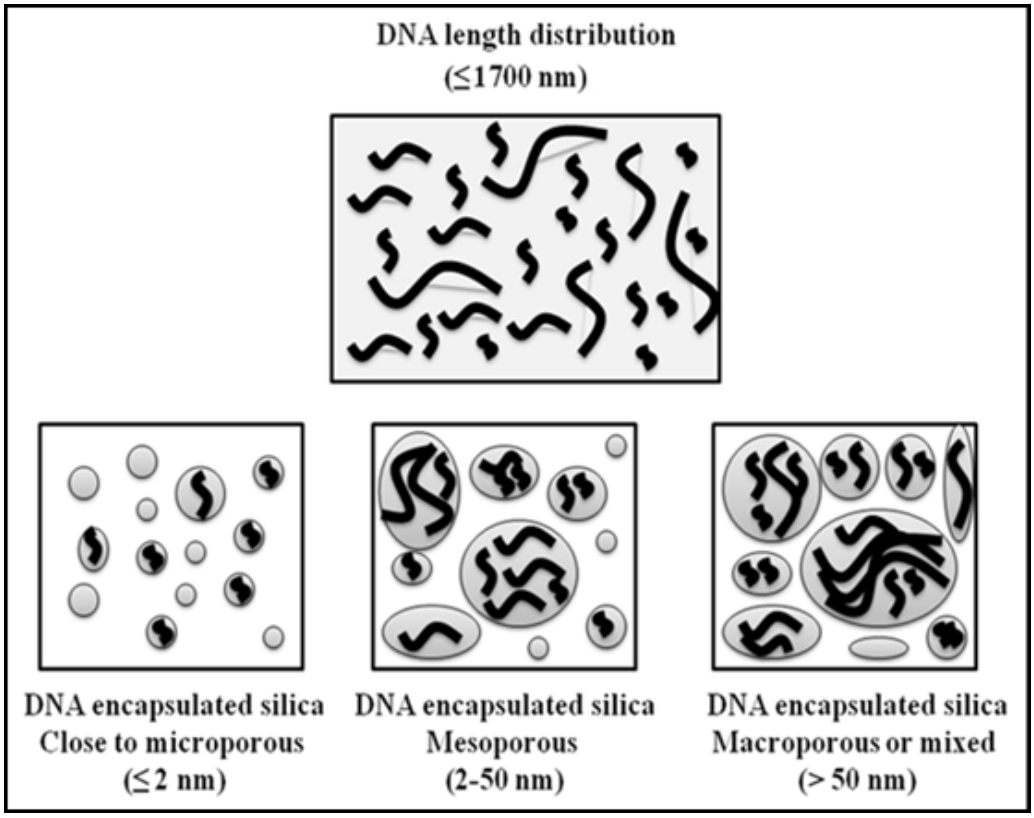

Figure 3.13 Representative schematics for encapsulation of DNA in microporous, mesoporous and mixed silica host, illustrating the differences in DNA holding capacity due to microstructural variations.

CHAPTER 4

DNA ENCAPSULATION IN HYBRID SILICA MODIFIED WITH AMINO-PROPYL TRIETHOXYSILANE (APS)

Pure silica matrices have potential to encapsulate DNA molecules in which the encapsulation by the physical confinement of the DNA molecules in the pores. In order to investigate the encapsulation of DNA in chemically modified silica, hybrid sol-gels including chemical functions for DNA anchoring were prepared. For this purpose, amino-propyltriethoxysilane (APS, $SiCH_3H_6NH_2(OC_2H_5)_3$) has been employed to chemically modify the silica. Molecular structure of APS was shown in Figure 2.1. APS is also an organic Si-precursor and its molecular structure resembles the structure of TEOS, containing three reactive alkoxy groups (ethoxy, $-OC_2H_5$). But in the APS, the forth side group is replaced with a hydrocarbon chain carrying amine ($-NH_2$) group. In TEOS, all side groups are ethoxy. In APS, the amine carrying side group is covalently bonded to silicon and does not react or decompose in aqueous environment. So, when condensed in presence of water, APS may form molecular level composite silica, through hydrolysis and condensation reactions. In the composite silica, amine ($-NH_2$) groups become an intrinsic part of the silica (Si-O-Si) network. Such hybrid silica structure may strongly bind DNA molecules due to the protonated ($-NH_2$) groups which provide electrostatic affinity for the negative charge carrying sugar-phosphate (PO_4) backbone of DNA.

One problem for using APS is related to its effect on chemistry of sol-gel reactions. Aqueous solutions of APS are highly basic and the control of the hydrolysis and condensation reactions might be difficult. In addition, highly basic nature of APS may inversely affect the molecular structure of DNA itself.

The processing parameters should be optimized for producing efficient and feasible DNA encapsulation matrices. In this chapter, characterization of DNA containing APS modified TEOS derived silica is presented. First, the gelation behavior of hybrid silica gels made up from pure APS has been discussed. Then, gelation behavior of TEOS-APS combinations and effect of APS addition on chemical/microstructural properties of silica and on DNA encapsulation efficiency have been investigated. At last, accessibility of DNA in TEOS derived and hybrid (TEOS:APS) silica were compared.

4.1 Gelation behavior of APS derived silica

In order to see the gelation behavior of APS, pure APS derived gels were prepared by simply dissolving APS in EtOH in presence of an acid catalyst. The formula was APS:EtOH:HCl (5:5:1, vol%) instead of previously prepared TEOS:EtOH: HCl (5:5:1, vol%). The preparation method was similar to TEOS derived silica, presented at very beginning of the results section. The only difference is using APS as silica forming precursor instead of TEOS. Acid catalysis mechanism was used to compare the gelation behavior of gels just to achieve a controllable gelation rate in each silica gel. Visual examinations showed that pure APS derived sols were turbid upon hydrolysis and the final gels were not transparent as the TEOS derived pure silica gels. This remarkable difference in the appearance was due to different chemical nature of APS precursor. As presented earlier, pure TEOS derived sols have an acidic nature during gelation and final pH was found to be 4.6. However, due to strong basic nature of APS, the final pH was 8.2 for pure APS derived sols. Gelation times of each gel were also consistent with the final pH values. TEOS derived sols were gelled in 20 days; however it was much faster in APS derived gel which gelled in 2 min with high opacity.

It is clear that gelation of silica from pure APS precursor is possible and gelation occurs faster compared to TEOS derived silica samples. However, the gelation rate and final pH of the sol might not be compatible for the conditions needed for DNA encapsulation. The gelation rate can be modified and adjusted for combination of APS and TEOS when both used as silica forming precursors. In the coming section, the effect of APS amount on gelation behavior of TEOS derived silica is investigated. This time formulations also contained DNA incorporation.

4.2 Gelation behavior of DNA containing hybrid (TEOS-APS) silica

The accelerated rate of sol-gel reactions in APS derived gels compared to pure TEOS derived gels is due to the basic amine ($-NH_2$) groups increasing the condensation rate and efficiency. This was an expected result . However, the effect of APS modification to TEOS derived silica formation is may be different in DNA presence. Thus, in order to investigate the effect of APS precursor on gelation of DNA containing TEOS derived silica, different formulations with varying APS/TEOS ratio (0.01, 0.05 and 0.5 - volume based) were prepared. The formulations are given in Table 4.1. The DNA amount was identical in each formulation. These formulations were chosen so that the results were comparable to the DNA containing TEOS derived gels (Table 3.7). The gelation of silica sols were accelerated with increasing APS/TEOS ratio in the presence of DNA. DNA in its buffer solution has also a basic nature so that there was not any inverse effect on gelation. The final pH was basic for each and increased from 8.2 to 8.5 and 10 with increasing APS/TEOS ratio from 0.01 to 0.05 and 0.5, respectively. The gelation times were similar and it was around 1 min due to high basicity.

Therefore, DNA encapsulation in APS modified TEOS derived gels seem to be possible. However, this requires optimization of the composition which eventually governs the gelation rate. The chemical and microstructural properties of the new hybrid (TEOS-APS) gels are provided in the coming section.

Table 4.1 Sol-gel formulations, final pH and gelation times of DNA containing hybrid (TEOS-APS) silica gels.

Sol –gel formulation (volume based) (Et-OH : HCl: $DNA_{[TE]}$)	**APS/TEOS**	**Description**	**Final pH**	**Gelation (min)**
5 : 1 : 4	0.01	Acid/base catalyzed APS modified silica containing DNA	8.2	1
5 : 1 : 4	0.05	Acid/base catalyzed APS modified silica containing DNA	8.5	1
5 : 1 : 4	0.5	Acid/base catalyzed APS modified silica containing DNA	10	1

4.3 Effect of APS on chemical properties Si-O-Si network

The APS modification is critical for the stability of encapsulated DNA molecules which is strongly controlled by the porosity and chemical structure of the silica network. Accordingly, NMR and BET techniques were used to characterize the hybrid (TEOS-APS) silica gels. The effect of presence of APS to chemical nature of silica network is shown by the NMR spectra in Figure 4.1. This data only have qualitative meaning and shows the ^{29}Si NMR spectra of TEOS, APS or TEOS-APS derived silica networks without any DNA addition. The formulations (silica precursor: EtOH: HCl) were as 5:5:1 for TEOS and APS derived silica.

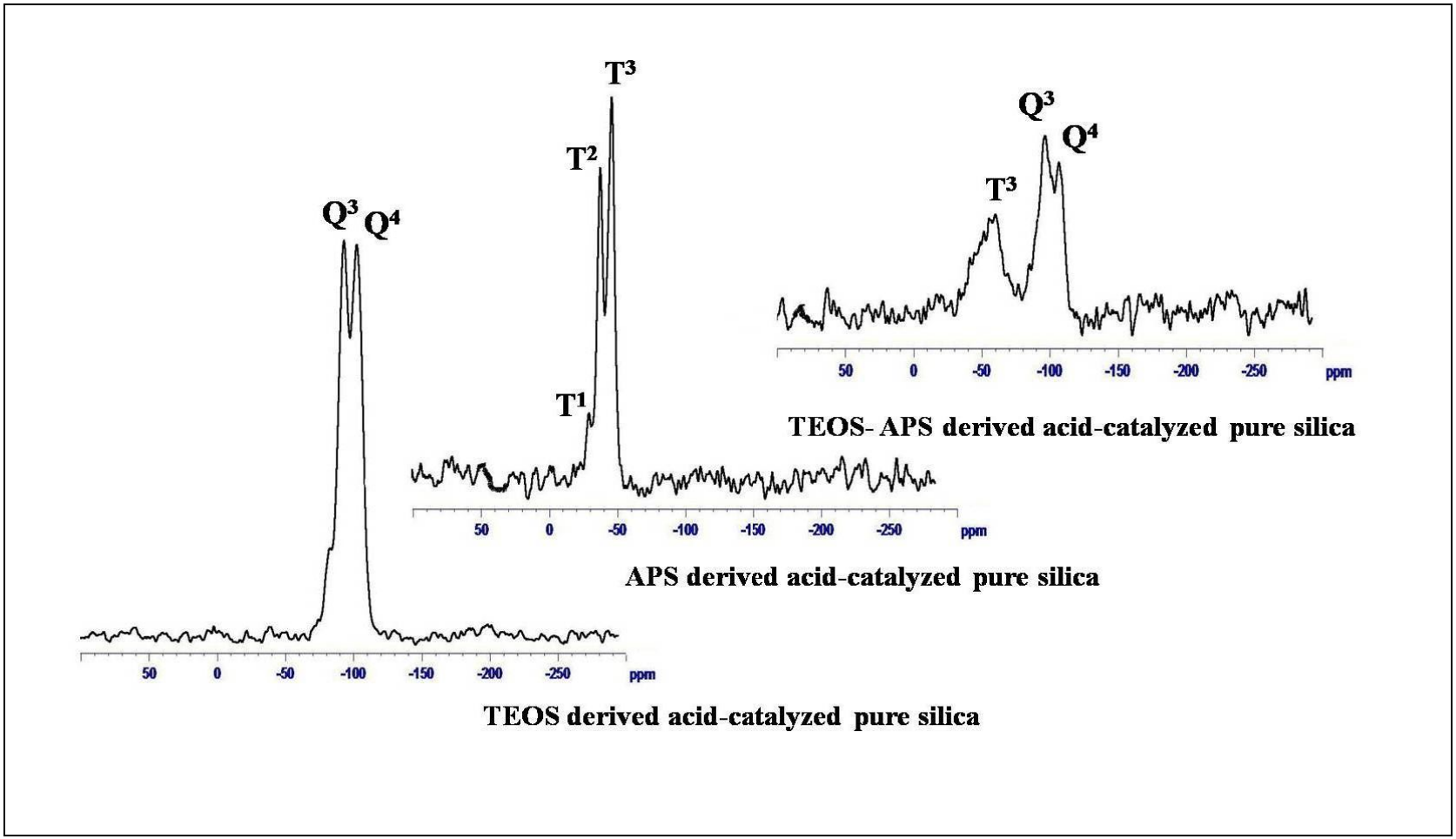

Figure 4. 1 ^{29}Si NMR spectra of acid-catalyzed TEOS, APS derived and hybrid (TEOS-APS) silica

For the hybrid (TEOS-APS) silica APS/TEOS ratio was 0.5 and EtOH and HCl content were identical with the pure TEOS and APS derived silica. The relative percentages of the chemical species are summarized in Table 4.2.

The chemical species for TEOS derived silica are Q^1 ($Si(OSi)(OR)_3$ at -84 ppm, Q^2 ($Si(OSi)_2(OR)_2$ at -90 ppm, Q^3($Si(OSi)_3(OR)$ at -100 ppm and Q^4 ($Si(OSi)_4$). [Brennan and Hartman (1999)]. APS derived silica has additional peaks (T sites-T refers to tri-substituted siloxane bonds). These resonance peaks are due to the difference in environment of silicon atoms originating from the presence of APS. These two resonance peaks are assigned as T^2 ($SiO_2(OH)$-R) at -57 ppm and T^3 (SiO_3-R) at -65 ppm [Chong and Zhao (2003)].

NMR analyses show that there may be unreacted precursor molecules in pure APS derived gels. Pure TEOS derived silica has only Q^n, and APS derived silica has only T^n species designating a minimal silica network formation efficiency. In the hybrid gels, when TEOS is modified with APS, the Si-O-Si network formation efficiency is at moderate level. This is evident from the decrease in the amount of Q^4 species from 43 to 33 % when a hybrid (TEOS-APS) silica is formed. Therefore, it can be concluded that APS modification still allows the Si-O-Si network formation, however it decreases the network formation efficiency. The relatively high condensation rate of silica as a result of high basicity (due to –NH_2) leaving unreacted –OH or –C sites during condensation reaction.

Table 4.2 Relative Q^n-T^n species of pure TEOS, APS derived and hybrid (TEOS-APS)silica

Description	**Q^4**	**$\sum Q^n$ ($n \neq 4$)**	**$\sum T^n$**
Acid-catalyzed TEOS derived pure silica	43	57	-
Acid-catalyzed APS derived pure silica	-	-	100
Acid-catalyzed TEOS-APS derived silica	33	42	25

4.4 Effect of APS on chemical and structural properties of DNA-containing silica

The chemical and structural properties of DNA-containing hybrid (TEOS-APS) silica structures with varying amount of APS addition (given in Table 4.1) were also examined by NMR and accompanying BET analyses. Figure 4.2 shows the NMR spectra of hybrid silica with APS/TEOS=0.01, 0.05 and 0.5. The tabulated structural information on the relative percentages of the chemical species for each hybrid network is also given in Table 4.3. Q^4 percentages can be used to compare the degree of silica network formation for each hybrid gel. The Q^4 species corresponding to $Si(OSi)_4$ was found as around 54% for the hybrid gels with APS/TEOS=0.01 and 0.05. Since the added APS amount was so small, formation of silica network was not so much affected. Total T^n species was 12% and 17% for silica with APS/TEOS=0.01 and 0.05, respectively. However, with further increase in APS amount (APS/TEOS=0.5), there were a remarkable decrease in the number of Q^4 species and an increase in the number of T^n species. The total Q^4 was 22% and total T^n species was 32% for the hybrid gel with APS/TEOS=0.5. This is again in agreement with the resultant hybrid silica formed without any DNA. APS precursor decreases the network formation efficiency. An incomplete silica network forms with increasing APS amount.

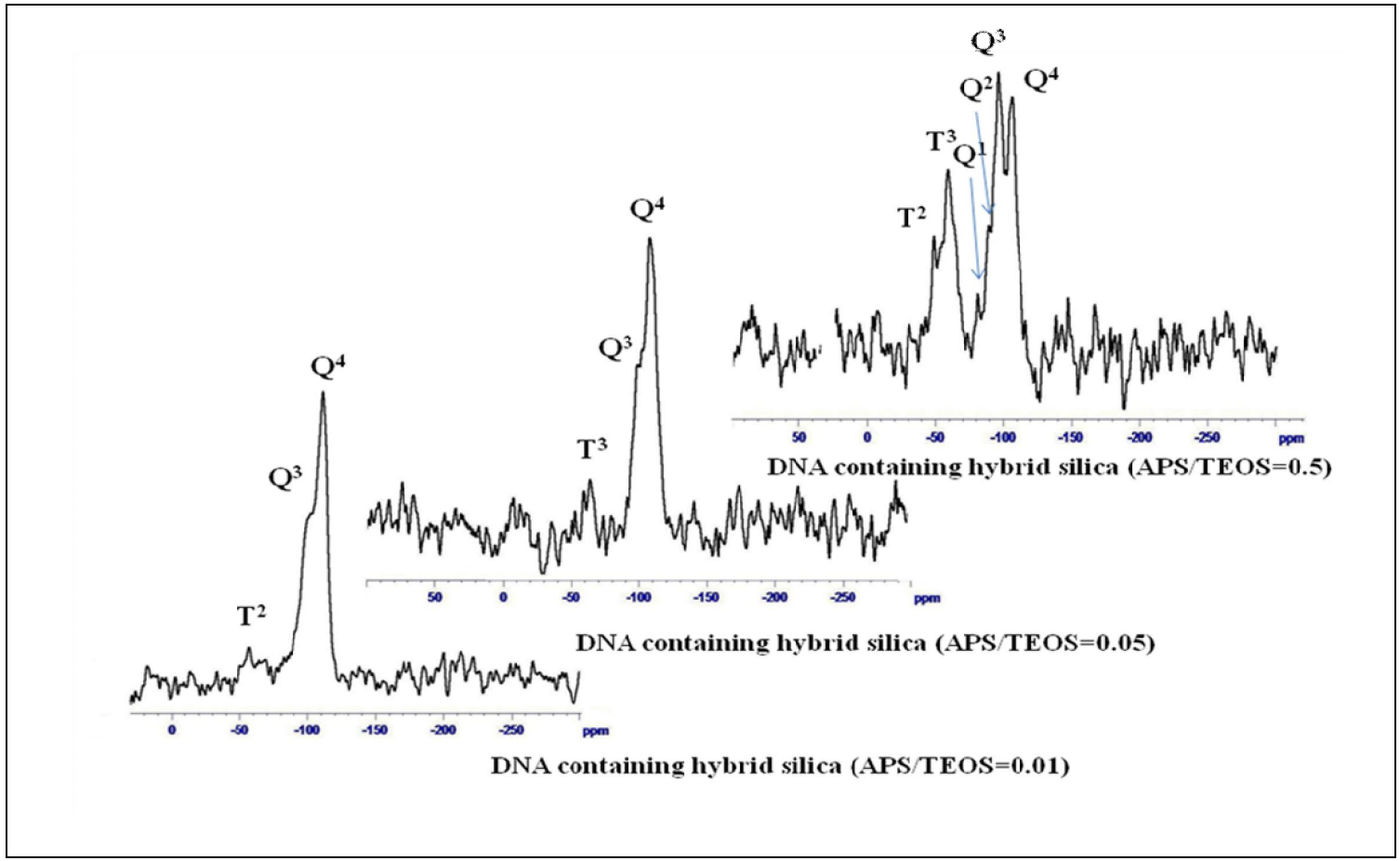

Figure 4.2 ^{29}Si NMR spectra of DNA containing hybrid (TEOS-APS) silica gels with APS/TEOS=0.01, 0.05 and 0.05 (volume based ratio).

Table 4.3 Relative Q^n -T^nspecies of DNA containing hybrid (TEOS-APS) silica gels with APS/TEOS=0.01, 0.05 and 0.5 (volume based ratio)

Description	Q^4	$\sum Q^n$ (n≠4)	$\sum T^n$
DNA doped acid/base catalyzed silica (APS/TEOS=0.01)	54	34	12
DNA doped acid/base catalyzed silica (APS/TEOS=0.05)	54	29	17
DNA doped acid/base catalyzed silica (APS/TEOS=0.5)	22	46	32

Figures 4.3-4.4 illustrate the gas adsorption-desorption isotherms and pore size distributions for DNA containing hybrid (TEOS-APS) gels with varying amount of APS. In Table 4.3 porosity characteristics of samples are summarized. There is a gradual raise in mesopore volume with increasing amount APS and pore volume reaches from 4 cm^3 to 4.2 cm^3 and 17.6 cm^3. The increasing mesopore volume with the APS modification indicates that each DNA containing matrix has a highly porous nature. In relation to this, one may expect a decrease in surface area. But, the surface area also increases with increasing APS amount. It was 4.7 m^2/g for silica with APS/TEOS=0.01, 13 m^2/g and 62.5 m^2/g for silica with APS/TEOS=0.05 and 0.5, respectively. This points out that APS modified hybrids silica may have a macroporous nature (or mixed 35-100 nm). The BET technique and the gas adsorption equipment is not capable of analyzing porosity when the average pore size reaches to macroporous range, i.e. when average is pore size is bigger than 50 nm. Therefore it can be said that each silica has highly porous structure with pores majorly bigger than 40 nm and pore sizes increases with increasing APS amount. The average mesopore size was around 2±1 nm for each silica. The remarkable increase in average pore size is also evident from the kicks in the adsorbed gas amount at relatively high pressures at around P/P_0=0.9 in the adsorption isotherms given in Figure 4.3. The structural properties of DNA encapsulated silica is strongly affected by the presence of APS precursor. In summary, APS addition caused lower condensation efficiency and inhibited silica network completion. It also leads to more porous silica structure.

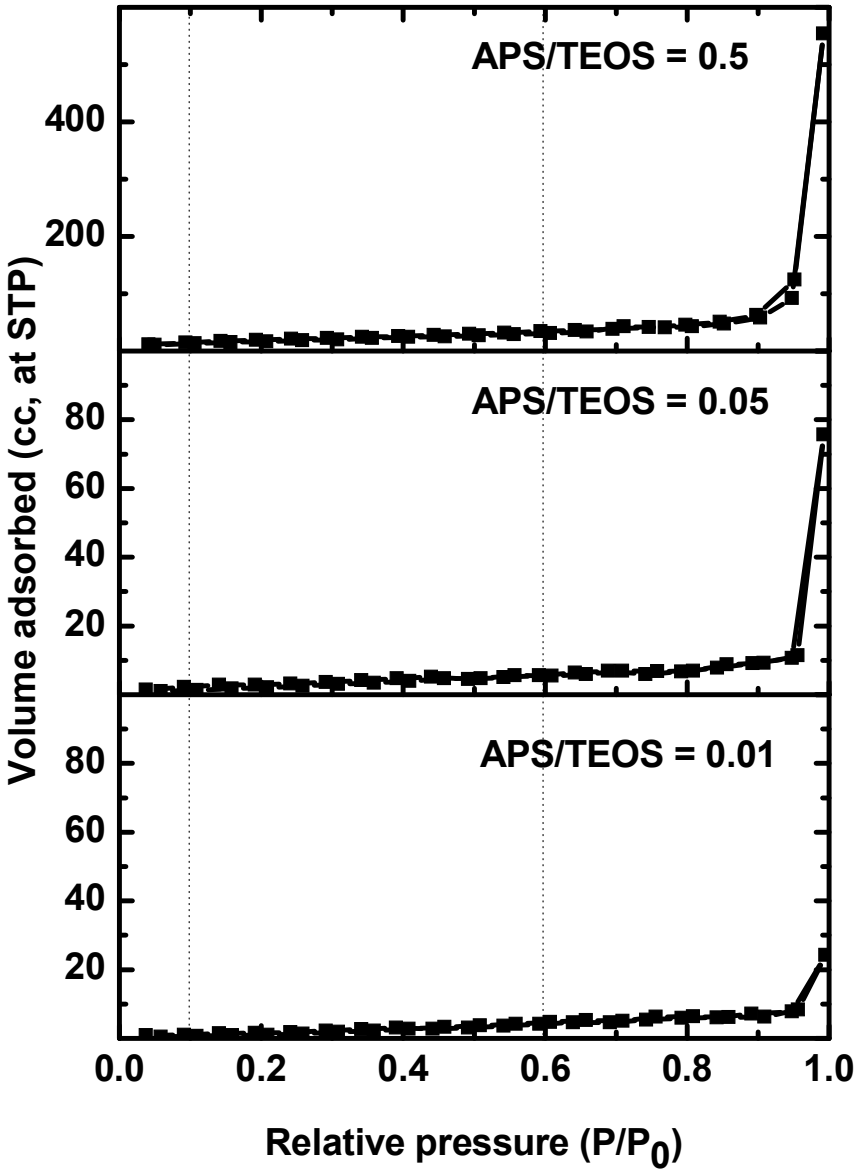

Figure 4.3 Adsorption-desorption isotherms of DNA containing hybrid (TEOS-APS) silica gels with APS/TEOS=0.01, 0.05 and 0.05 (volume based ratio).

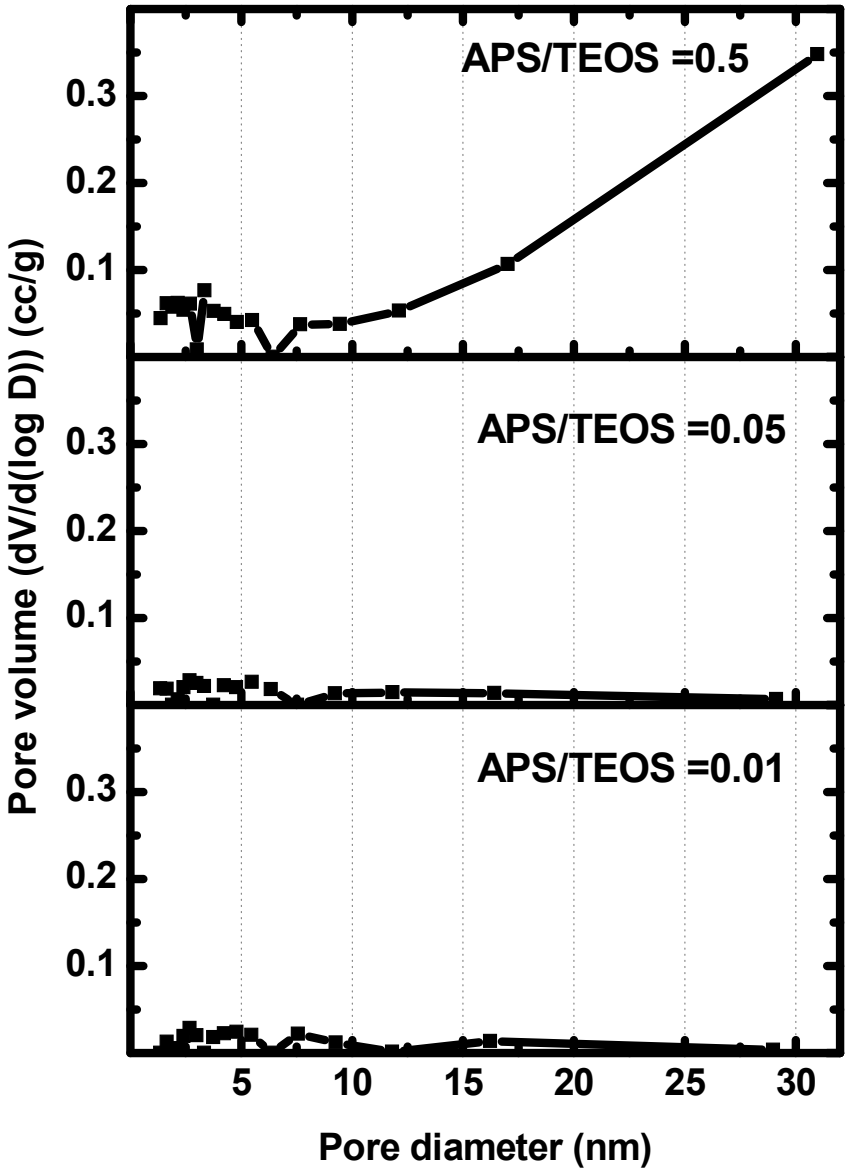

Figure 4.4 Pore size distributions of DNA containing hybrid(TEOS-APS) silica gels with APS/TEOS=0.01, 0.05 and 0.05 (volume based ratio).

Table 4.4 Porosity characteristics (average pore size, surface area, and mesopore volume) of DNA containing hybrid (TEOS-APS) silica gels with APS/TEOS=0.01, 0.05 and 0.5 (volume based ratio).

Sample description	Average pore size (nm)	Surface Area (m^2/g)	Mesopore volume (cc) (P/P_0 =0.1-0.6)
Acid-base catalyzed (APS/TEOS=0.01)	2.5±1	4.67	3.95
Acid-base catalyzed (APS/TEOS=0.05)	2.4±1	13.02	4.22
Acid-base catalyzed (APS/TEOS=0.5)	2.2±1	62.47	17.58

4.5 DNA encapsulation efficiency in hybrid (TEOS-APS) silica

In order to investigate the DNA encapsulation efficiency in APS-modified silica matrices, DNA-containing hybrid silica powders with different APS:TEOS amount were characterized by optical analyses. This was again achieved by UV-vis absorption analyses; while using ethidium bromide (EtBr) as a optical marker that can intercalate into DNA strands that are encapsulated within the porous silica matrices.

Figure 4.5 illustrates the UV-vis spectra of the supernatant EtBr solutions that have been in contact with DNA containing APS-TEOS hybrid gels for 3 h. The formulations of these hybrid silica powders containing identical amount of DNA are given in Table 4.1. The inset pictures in Figure 4.5 show the color changes for the hybrid silica powders after 3 h. The DNA encapsulation was interpreted similar to previous studies, by comparing the change in intensity for the absorbance band at 285 nm, which is attributed to absorbance by EtBr molecules. The comparison can be performed for initial state (t=0) of the EtBr solution and after exposure of EtBr solution to hybrid silica powders for 3 h. The intensity of this particular band for the EtBr solution (labeled as reference EtBr in the Figure 4.5) corresponds to the initial state, i.e. before introducing the DNA containing silica powder in EtBr solution.

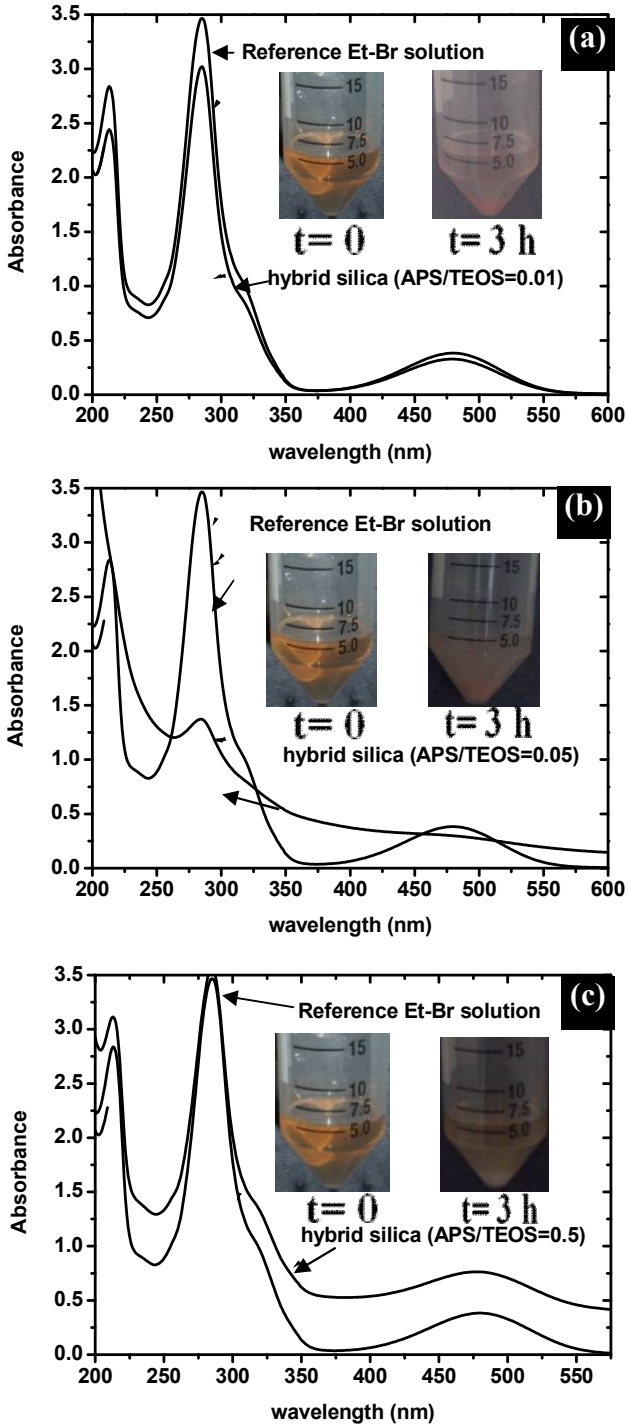

Figure 4.5 UV-vis absorption spectra for supernatant EtBr solutions exposed to silica gels with APS/TEOS : a)0.01, b) 0.05 and c) 0.5 (volume based ratio). Note that each powdered silica was exposed to pure EtBr solution (at 10 μg/ml concentration) for 3 h. Insets are photos of EtBr solution at initial stage after interaction with silica powders for 3h.

Since all hybrid gels are containing same amount of DNA, the decrease in the intensity of the 285 nm-band should be at the same or at least at a comparable extent upon intercalation of the EtBr molecules into the DNA strands encapsulated within the silica matrices. However, an intensity decrease for this band is only observed for the lowest amount of DNA containing hybrid silica powder with APS/TEOS=0.01 was apparent. This low APS-containing hybrid silica was also the only powder exhibiting a color change (from white to pink) after 3 h. These observations indicate that EtBr intake occurs only for this specific formulation suggesting the presences of encapsulated DNA.

For the other hybrid powders with higher amount of APS, of no distinct color change for the powders was observed. This means there was any Et-Br intercalation in to encapsulated DNA in these matrices or there was no encapsulated DNA in these hybrid silica matrices at all. This may be to several reasons. One possibility is related with assessment method (Et-Br adsorption method) itself which relies on intercalation of EtBr to DNA. But this physical interaction between DNA-and Et-Bt is very similar to the interaction of APS with DNA. EtBr molecules intercalate into DNA base pairs. On the other hand, APS attaches to phosphate (PO_4) backbone of the DNA molecules through an electrostatic affinity due its protonated amine (NH_2) groups. Therefore, event tough there are some encapsulated DNA strands within hybrid silica powder they may be not free for EtBr intercalation and forming complex with amine groups. But, there is another anomalous finding in the UV-vis spectra of Figure 4.5. The intensity of the 285 nm band of the supernatant EtBr solutions after 3 h are higher compared to their initial absorbance values. This can be only possible when some species (most likely organic) from the matrices transfer to EtBr solution leading to the unanticipated increase in the 285 nm absorption. The reason is obvious from the structural analyses by NMR. For higher amount of APS addition, the Si-O-Si network formation efficiency is very low. This hybrid gels are not stable and dissociates in aqueous environment. This means the encapsulating porous network loses its structurally integrity and collapses by aqueous attack.

4.6 DNA leach-out tests

In order to confirm the DNA encapsulation in TEOS derived and in hybrid (TEOS-APS) silica matrices, leach-out tests were applied to previously prepared silica samples. Sample formulation for TEOS derived silica was the one given in Chapter 3-Table 3.4 (HCl/TE= 0.2). The hybrid silica formulation was the one with APS/TEOS=0.01 (Table 4.1). The DNA amounts were identical for both samples. DNA leach-out from silica depends on the scale of porosity. As the pore size becomes smaller, DNA leach-out is expected to be more difficult. TEOS derived silica was mesoporous (5.6±1 nm averagely) and hybrid silica was macroporous (>50 nm averagely). Thus, one should expect DNA leach-out to be easier in hybrid silica. In order to investigate the leach-out behavior of DNA in these matrices, DNA-containing silica powders were emerged in 0.05 M TE buffer solution for 2h, 4 h and 2 days. Later on, silica powder containing TE buffer solutions were centrifuged at 10000 rpm for 5 min. Supernatant buffer solutions were separated and UV-vis absorption spectra of each solution were recorded. In order to analyze the leach-out species, UV-vis spectra of pure 0.05 M TE buffer was also recorded. Resultant UV-vis absorption spectra of supernatant solutions can be seen in Figure 4.6.

In Figure 4.6a, UV-vis absorption spectra of supernatant buffer for DNA containing TEOS derived silica for 2 h, 4 h and 2 days compared to pure TE buffer are given. There was not a remarkable absorption around 260 nm after 2 h, 4 h or 2 days indicating that no DNA was leached into the buffer solution. The only difference was the increase in absorption by time. It may be due to leaching of ionic species in silica into the solution. In Figure 4.6b, UV-vis absorption of supernatant TE buffer for DNA containing hybrid (TEOS:APS) silica for 2 h, 4 h and 2 days are compared to pure TE buffer. Similar to TEOS derived DNA containing silica, there was not any absorption band around 260 nm characteristic to DNA for all times.

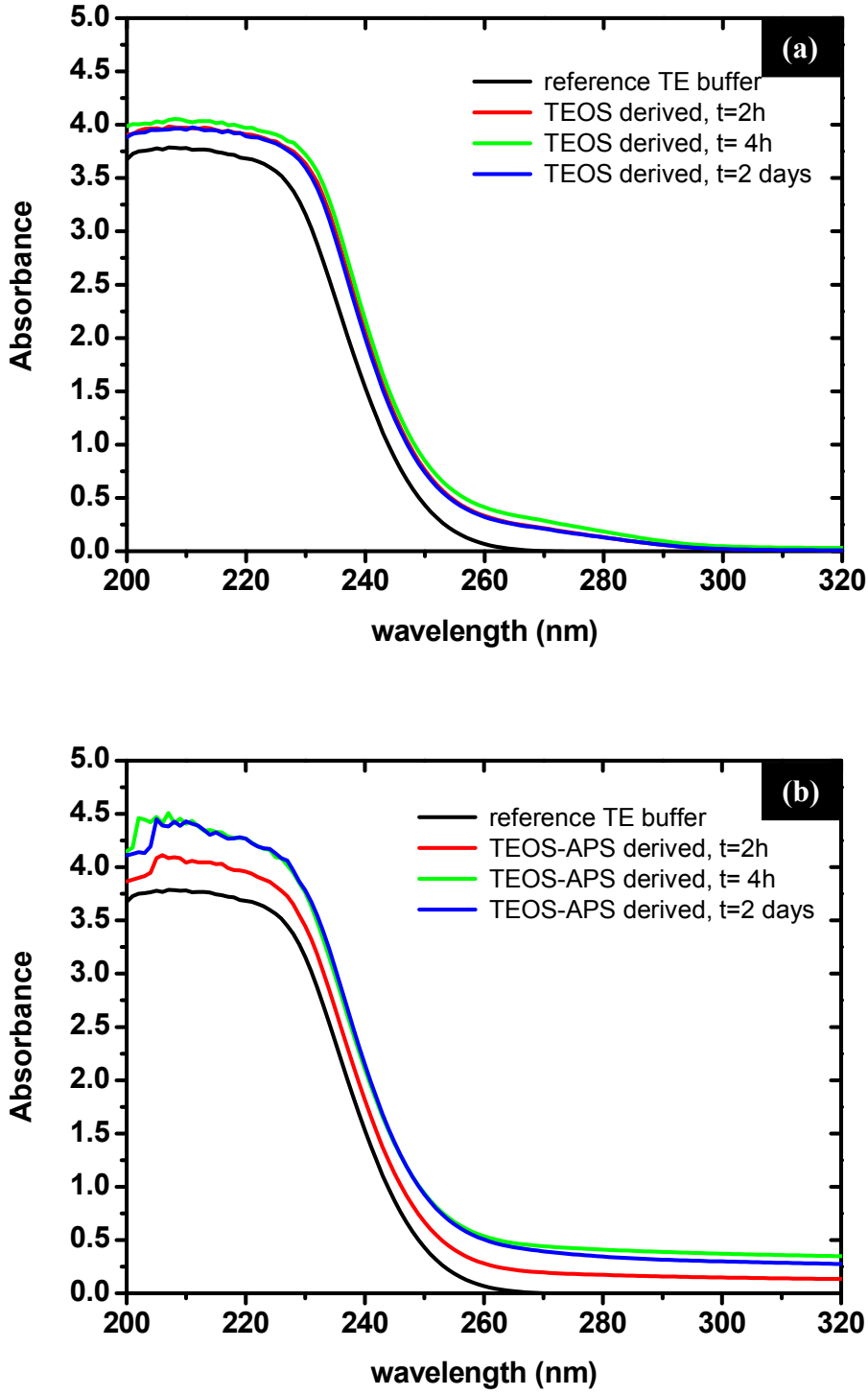

Figure 4.6 UV-vis absorption spectra showing DNA leach out kinetics for 2 h, 4h and 2 days from **(a)** TEOS derived silica (HCl/TE=0.2) and **(b)** Hybrid silica (APS/TEOS=0.01).

Shortly, it is apparent that DNA molecules were trapped in porous TEOS derived and hybrid silica microstructures up to 2 days. Results showed that DNA did not leach out into the buffer solution from both. This explains the effect of amine groups fixing DNA in the hybrid silica matrix. But, those DNA molecules were not functional.

In summary, chemical functionalization of the silica matrix with APS might be a promising processing way for improved encapsulation of DNA molecules just beyond a physical confinement in the pores. But, addition of APS as a DNA linking agent onto silica should be kept in small percentages. Higher amount of APS chemically modifies the network, but greatly hamper the completion of inorganic Si-O-Si network, reducing its chemical durability and inducing complete dissociation in aqueous environment.

CHAPTER 5

SUMMARY AND CONCLUSIONS

A sol-gel processing route for encapsulation of DNA molecule in silica was established. Commercially available crude DNA–double stranded salmon sperm DNA– was employed as a model DNA molecule. Pure and DNA-containing silica gels in powder form were produced by hydrolysis and condensation of an organic silica precursor (TEOS) catalyzed at different conditions. DNA encapsulation is achieved by incorporation of DNA containing TE-buffer solutions to sol-gel mixture prior to gelation, allowing entrapment of DNA molecules in the porous Si-O-Si network formation during gelation and drying.

The effects of i) sol-gel chemistry (catalyst type/concentration) and chemical modifications of silica network ii) DNA amount on chemical and microstructural properties of the resultant silica networks and on DNA encapsulation efficiency were investigated by combination of different analytical techniques. These include chemical investigations by NMR, microstructural investigation by gas adsorption techniques (BET), and optical analyses by UV-vis spectroscopy. In addition a simple, UV-vis spectroscopy-based analytical testing method for assessment of DNA encapsulation efficiency has been proposed. This method provides semi-quantitative measure for presence of DNA in sol-gel derived silica matrices by monitoring the kinetics and the extent of intercalation of an fluorescent tag (ethidium bromide) into the DNA-containing silica gels.

In order to determine the chemical and microstructural variations caused by the sol-gel chemistry, DNA containing silica matrices under different catalyst concentrations were prepared; while keeping the DNA content identical.

Performing the gelation at alkaline conditions affected the chemical and structural properties of silica matrices. As the basic catalyst amount was increased, condensation reaction was accelerated providing a more condensed silica network with high gelation rates. This also changed the porosity of the silica microstructure. Pore volumes and the average pore size of these matrices increased remarkably with an increase in the basic catalyst concentration. The chemistry-derived microstructural variations control the DNA encapsulation efficiency in these silica matrices and the encapsulation efficiency (or functional DNA holding capacity) for the sol-gel derived silica matrices strongly depends on the size of the porosity attained.

An efficient encapsulation requires a size match between the pore size and length of the DNA molecules without chemical interaction between DNA and silica that can affect DNA's conformation. Higher level of DNA encapsulation is observed for the mesoporous silica matrices with bigger pores (20 nm and bigger) that were obtained by basic catalysis conditions. In the case of microporous silica with much smaller pores (2 nm and smaller), which is formed under acid-catalysis, only marginal DNA encapsulation can be achieved. If the average pores size is too small and reaches to the microporous size level, only undersized DNA fragments with appropriate sizes matching the pore size become entrapped.

Sol-gel chemistry studies also revealed some information about the DNA encapsulation mechanism. The NMR analyses showed that the interaction between encapsulated DNA and Si-O-Si network is not through any chemical bonding; and the encapsulation occurs by physical entrapment/confinement in the pores. EtBr absorption and associated optical analyses suggested that the encapsulated DNA molecules were preserved mostly in their original state, i.e. in their native double stranded form, without any deterioration due to chemical reactions during gelation of silica network by hydrolysis and condensation.

Presence of DNA increases the inorganic Si-O-Si network completion (condensation) efficiency during gelation of TEOS and this is more promoted with increasing DNA amount. Additionally, the scale of porosity and the average pore size of the resultant silica increases with amount of DNA-TE buffer solution in the starting sol-gel formulation.

The effect of DNA amount is somewhat analogous with the effect of catalysis type. At overall, a DNA addition pushes the gelation reaction conditions to the alkaline pH regions. This is due to basic nature of DNA molecules itself with hydroxyl (-OH) binding active sites on sugar-phosphate backbone. Alkaline catalysis conditions also promote gelation efficiency and also favoring formation of bigger forms, which in turn provides a higher DNA encapsulation potential for the resultant silica matrices. But, there is no absolute and direct correlation between the amount of DNA in the starting sol-gel formulation and final DNA amount present in encapsulated from in the final gelled silica. In other words, the limit for total DNA that can be encapsulated again determined by structural characteristics mainly by the average pore size governed by the gelation chemistry.

Aqueous sol-gel processing can be adapted to allow modification of the silica network with chemical functional groups such as amine ($-NH_2$) groups to achieve a chemical interaction between encapsulated DNA and silica matrix. This can be accomplished effectively in hybrid silica systems formed by simultaneous hydrolysis and condensation of amine-containing organic precursor APS together with TEOS. However, this hybrid approach leads to more open and more incomplete Si-O-Si network formation; making the resultant silica network more vulnerable to aqueous attack. Such DNA containing hybrid silica simply dissociates due to dissolution of silica network and does not serve as durable cage for encapsulation of DNA molecules.

FUTURE WORK

In the thesis, the usability of sol-gel derived silica matrices for DNA encapsulation was shown. The DNA encapsulation efficiency was mainly related to the porosity attained. Since the DNA molecules used for encapsulation studies were different in size, only the DNA molecules matching with the pores in size could be encapsulated. In this respect, as a future direction, double stranded DNA molecules that are identical in strand size can be used for encapsulation in silica matrices. In such systems, the correlation between encapsulated efficiency and average pore size can be established in a more systematic manner.

On the other hand, it was seen that the solution pH was one of the main factors controlling the gelation kinetics and the silica network formation. DNA was found to behave as a basic catalyst in silica formation. However, the mechanism behind that behavior is still not clear. Silicon NMR studies showed that DNA did not react with the silica matrix, but any of other chemical interaction (even tough it does not denature DNA) between silica network and DNA should be analyzed. This can be achieved by special phosphorous NMR.

The hybrid modification seemed to be ineffective due to increased gelation rate that does not allow silica network completion. Only three different amounts of APS modification were analyzed in the thesis. The effects of smaller amounts of APS modification on encapsulation mechanism and efficiency can be also determined in future studies.

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APPENDIX

1. Quantification of DNA amount

This section explains the details for quantification of DNA amount by optical analyses (UV-vis absorption analyses). This standard protocol was used in all analyses to determine the amount of DNA present in aqueous solution. This protocol is based on plotting a graphical calibration plot in terms of DNA amount (in μg/ml). and A_{260} absorbance values, for aqueous solutions with known DNA amount. For obtaining this calibration plot, first, different amounts of commercially supplied double stranded salmon sperm DNA powders were dissolved in TE-buffer. Each DNA containing buffer solution was then analyzed by UV-vis spectroscopy and absorption spectra in 200-320 nm range were obtained for each solution (as shown Figure App. 1a). The values of absorption maxima at 260 nm (A_{260}) characteristic for DNA absorption were then tabulated and a calibration line was plotted accordingly (as shown in App.1b) In Figure App.1b, the linear relationship between the DNA concentration and the UV absorption at 260 nm can be seen. The equation of this linear dependence was determined by using the best fitting function in Origin Pro8 graphic software as:

$$A_{260} = -0.05855+0.02932 \cdot x_{[DNA]} \qquad \text{Eq.(App. 1)}$$

where A_{260} is the measured absorbance at 260 nm and $x_{[DNA]}$ is the DNA concentration in (μg/ml). Throughout the thesis, this equation has been used to convert a measured absorption (A_{260}) to a representative DNA amount. The reason for using such calibration approach was to eliminate any experimental error in quantification studies.

The absorption (A_{260}) is directly related to the type and concentration of DNA in solution. For double stranded DNA (dsDNA), 50 μg/ml dsDNA corresponds to A_{260}=1 [Stephenson (2003)]. But, it is well known that this value may change due the sensitivity of the spectroscope or due to the presence of impurities in DNA. The plot in Figure App. 1b also shows that this concern is valid for the DNA used in this thesis. An A_{260} value of 1.25 for a DNA (in our case double stranded salmon sperm DNA) solution with a concentration of 50 μg/ml, is indeed different than the expected value of 1.

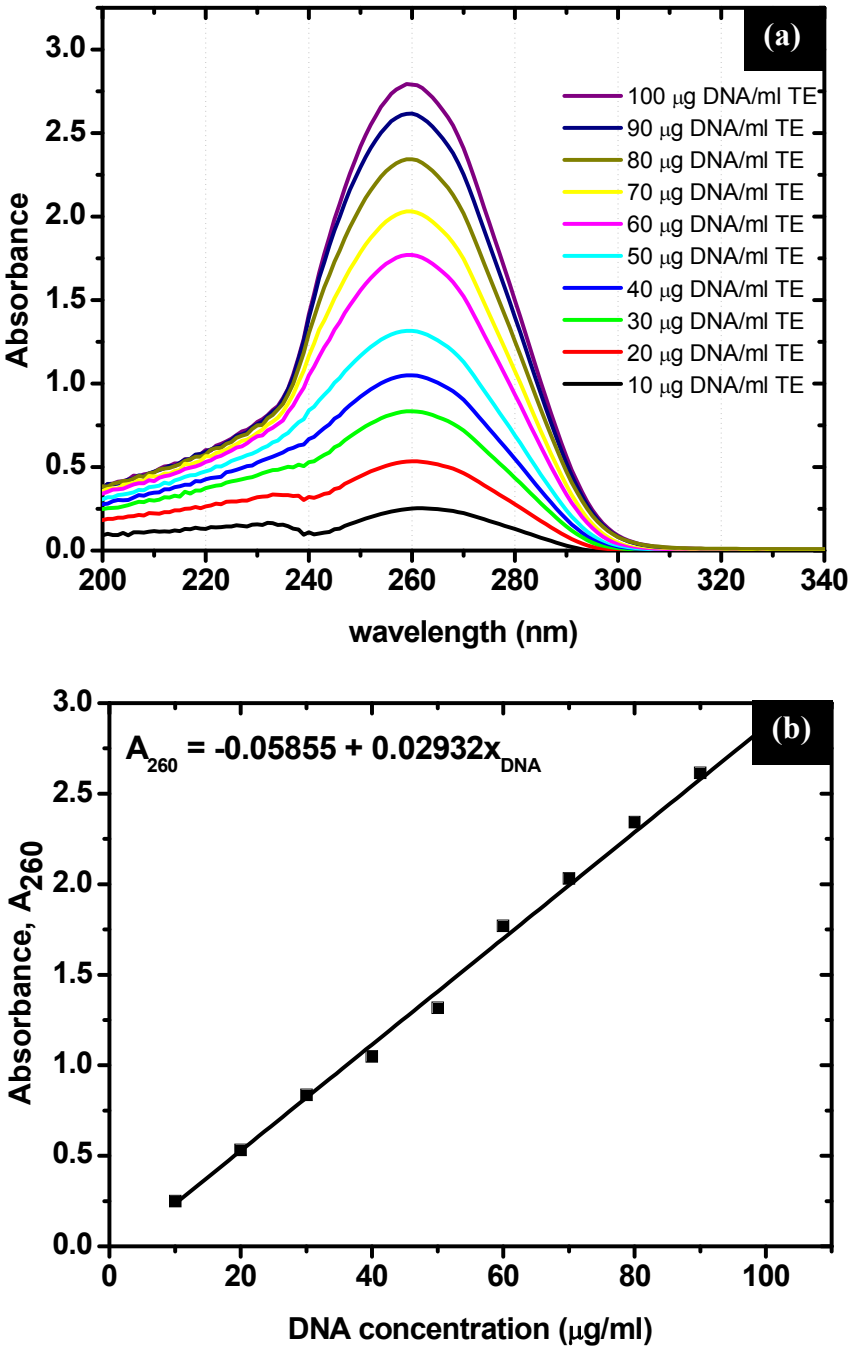

Figure App.1 a) UV-vis spectra of TE buffer solutions containing DNA at different concentrations (in μg/ml). **b)** The concentration dependence of A_{260} absorbance. The equation given in the graph is obtained by best fitting function in Origin Pro8 program.

2. Quantification of ethidium bromide (Et-Br) amount

A similar approach was followed to determine the Et-Br amount in any solution. In order to evaluate the characteristic absorption behavior aqueous solutions containing Et-Br, first the absorption spectra of water-based Et-Br solutions at different concentrations were obtained (as shown in Figure App.2a). There are two absorption bands which are characteristic to absorbance by Et-Br; one at 285 nm and the other at 480 nm. As the Et-Br concentration in solution increases, the intensity of UV absorption at these wavelengths also increases. The absorbance value for the broad peak at 285 nm was used in plotting the Et-Br calibration plot. By the use of best fitting function in Origin Pro8 program, the equation for linear dependence was then determined as follows:

$$A_{285} = 0.0782+0.3456 \cdot x_{[Et\text{-}Br]} \qquad \text{Eq.(App. 2)}$$

where A_{285} is the measured absorbance at 285 nm and $x_{[Et\text{-}Br]}$ is the EtBr amount in solution, in µg. In determining the Et-Br absorption amount to DNA containing silica gels, silica powders (50 mg) were exposed to 5 ml of Et-Br solution at 10 µg/ml concentration for 3 h. Later on, the solution containing DNA encapsulated silica powders were centrifuged and the powders were separated from the liquid (supernatant) solution. The supernatant solutions were analyzed by UV-vis spectroscopy. The intensity of the measured absorption peak (at 285 nm) was then plugged into calibration equation to quantify the Et-Br amount in the supernatant solution.

It is noteworthy to mention that pure silica was also tested by EtBr absorption; however, there were a very small absorbance due to the physical absorption on silica surfaces.

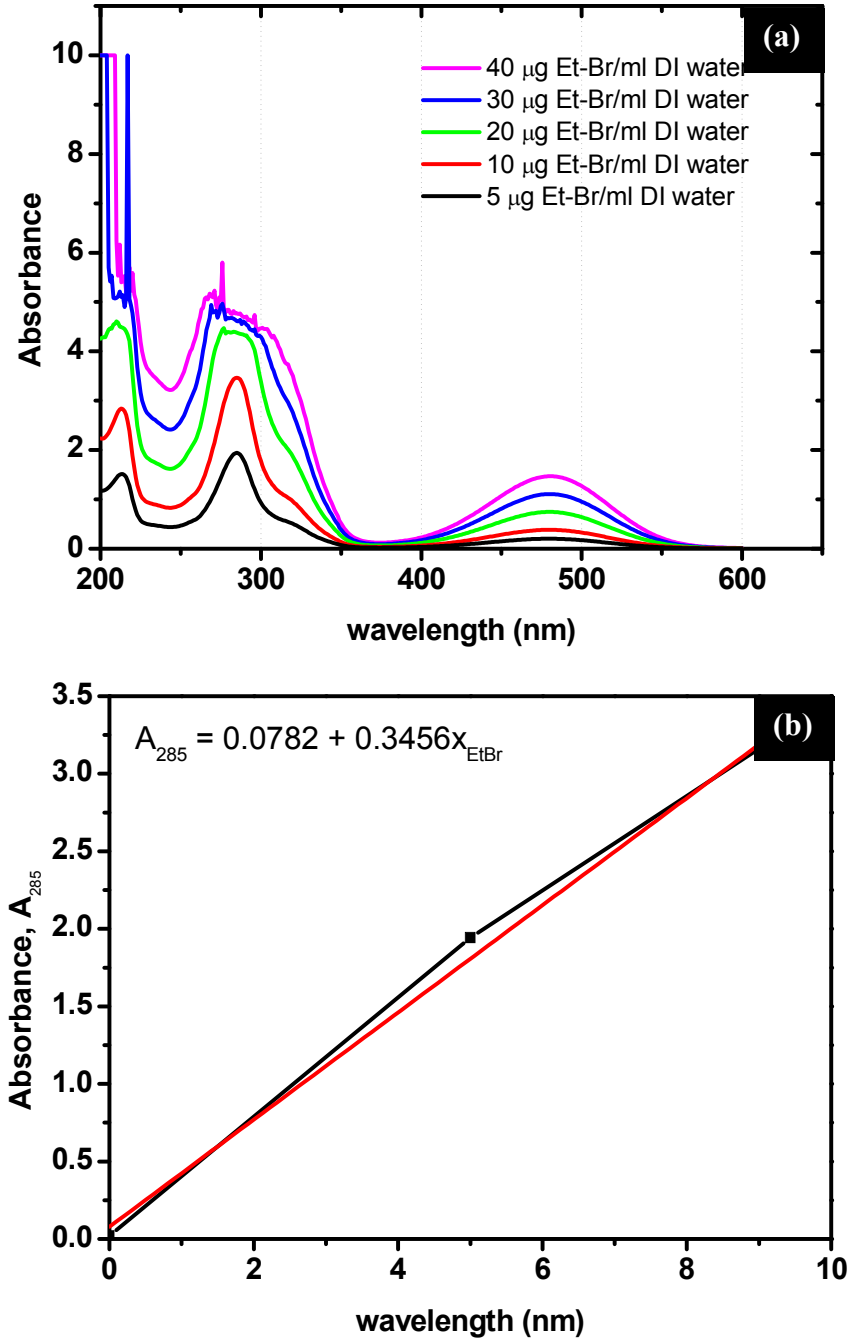

Figure App. 2 a) UV-vis spectra of aqueous ethidium-bromide solutions at different concentrations. Note that the baseline solution was water and it is extracted for each spectrum **b)** The concentration dependence of A_{285} absorbance. The equation given in the graph is obtained by best fitting function in Origin Pro8 program.