CHAPTER II

THEORY AND LITERATURE REVIEW

2.1 Tissue Engineering

As a result of donor tissue shortage and increasing number of organ-lost patients, tissue engineering has been of interest to medical personnal and biomaterial scientists for solving these problems. The definition of tissue engineering is the application of principles and methods of engineering and life sciences to obtain a fundamental understanding of structure-function relationships in novel and pathological mammalian tissue, and the development of biological substitutes to restore, maintain, or improve tissue functions, defined by NSF Workshop on Tissue Engineering [26]. The goal of tissue engineering is to surpass the limitations of conventional treatments based on organ transplantation and biomaterial implantation [27]. There are 3 principal therapeutic strategies for treating diseased or injured tissues in patients;

i) Implantation of freshly isolated or cultured cell,

ii) Implantation of tissues assembled *in vitro* from cells and scaffolds, and

iii) *In situ* tissue regeneration.

All strategies involve cells placing on or within scaffolds or three-dimensional (3D) matrices [28].

2.1.1 Scaffold on Tissue Engineering

Tissue engineering approach involves seeding the cells into the scaffold. The cells then proliferate, migrate and differentiate into specific tissue while secreting extra-cellular matrix components (ECM) required to create the tissue. Therefore, the

scaffold design is one of the essential factors in producing artificial tissues and organs [29].

There are many crucial requirements of a successful scaffold for tissue engineering application [1] as follows.

- Possessing interconnecting pores and having sufficient porosity (normally greater than 90%) for sufficient to replace or restore organ function of cells, diffusion of nutrients, and clearance of wastes.
- Having the proper pore size which each cell of tissue grows into implants (Table 1).
- Being made from material with biodegradability and biocompatibility.
- Non-toxicity
- Being easily sterilized to prevent infection.
- Having appropriate surface chemistry to favor cellular attachment, differentiation and proliferation.
- Possessing adequate mechanical properties to match the intended site of implantation and handling, not inducing any adverse response and, being easily fabricated into a variety of shapes and sizes.

Table 2.1 Type of cells and appropriate scaffold pore size.

Scaffold pore size (μm)	**Type of cells**	**Ref.**
15-100	Fibroblast	11
20-125	Fibroblast (adult mammalian skin)	30
100-350	Osteoblast	31
50-200	Hepatocyte	32

2.1.2 Scaffold Formation Techniques

As scaffold characteristics mentioned earlier, the scaffold fabrication technique should provide a scaffold with controlled porosity and pore size, since both are important in organ regeneration and also affect the mechanical properties of the scaffold. Many scaffold formation techniques have been studied to be appropriate for each material.

2.1.2.1 Thermally Induce Phase Separation (TIPS)

TIPS is a popular technique for scaffold fabrication. For theory, the polymer solution is cooled below the melting point of the solvent. After cooling, the polymer solution is separated into 2 phases: polymer-rich phase and polymer-poor phase. The latter phase is then removed by vacuum dry for several days until complete solvent sublimation giving highly porous within material. Up to now, TIPS technique was used to fabricate from both synthetic and natural polymers.

Poly(L-lactic acid) (PLLA) and poly(DL-lactic-co-glycolic acid) (PLGA) scaffolds had been fabricated by this technique [33]. The effect of polymer concentration, solvent/non-solvent ratio (a mixture of dioxane (solvent) and water (non-solvent) and quenching temperature on pore size were studied. It was found that quenching temperature was a significant effect. A slow cooling rate generated a macroporous open cellular structure, whereas a fast cooling rate gave a microporous closed cellular structure. Scaffold up to 90% porosity with pores of approximately 100 μm, had been produced.

For natural polymer dissolving in water, freeze drying term was used to call this technique instead. It was a bio-clean method having ice as a porogen, no any organic solvent remaining in scaffold after ice crystal sublimation.

Mahidally *et al.* [34] prepared porous chitosan by freeze-drying technique. Various forms of scaffold were produced including porous membranes, blocks, tubes and beads. The effect of chitosan concentrations and freezing temperatures were studied on pore size of scaffolds. They found that freeze-drying is a proper method

for fabrication various forms of chitosan scaffold. By varying freezing conditions, mean pore diameter, measuring by SEM photographs, were fabricated within the range 1-250 μm. However, the mechanical strength of porous scaffolds were lower than that of non-porous control.

Schoof *et al.* [35] developed a freeze-drying process to manufacture collagen sponges with a homogeneous pore for medical application. In production, the collagen suspensions were solidified under time- and space-dependent freezing condition. To control pore structure, the unidirectional solidification was applied during the freezing. They found that the unidirectional solidification and stationary freezing condition could produce collagen sponges with a homogeneous pore structure. The ice-crystal morphology and size could be adjusted between 20-40 μm by varying the solute concentration in the collagen suspension.

2.1.2.2 Solvent Casting and Particular Leaching

This method involves removing a porogen by solvent leaching. Pore size and % porosity of scaffold could be adjusted by controlling size and amount of the porogen.

Mikos *et al.* [36] prepared PLLA sponge using NaCl as porogen. The process involved 2 steps. The first step, PLLA was dissolved in chloroform and then added salt particles into polymer solution. After solvent evaporation, the polymer/salt composite was leached in water for 2 days to remove the porogen completely. They found that the pores exhibited high interconnectivity within 70wt% salt and above. Porosities in the range of 20-93% and pore sizes in the range of 30-120 μm have been achieved by this technique.

2.1.2.3 Internal Bubbling Process (IBP)

A new approach to fabricate porous matrices was developed from an attempt to extent the upper limit of pore size by freeze-drying. Chow *et al.* [37] produced open-pore matrixes by loading $CaCO_3$ crystals into chitin solutions. The mixture was cast to form gels and then submerged in 1N HCl solution to produce gaseous carbon

dioxide and $CaCl_2$ giving rise to highly open-pore matrixes. They found this method was suitable for chitin porous matrix formation because it could solve the problem of insolubility in most solvents of chitin. The obtained matrixes were uniform large pore sizes ranging from 100 to 1000 μm depending on the amount of $CaCO_3$ added into the chitin solution. In addition, the matrixes showed good water vapor permeability and water uptake ability with good mechanical strength.

2.1.2.4 Gas Forming

To avoid the use of organic solvent, a new approach involving gas as a porogen was developed to fabricate porous sponges [38]. The process began with the formation of poly (glycolic acid) (PGA), PLLA or PLGA solid discs using compression molding. The solid discs were saturated with CO_2 gas under high pressure (5.5 MPa) for 3 days at room temperature, and then the pressure was decreased to atmospheric pressure resulting in porous within the polymer matrixes. Scaffolds with porosities of up to 93% and pore size up to 100 μm could be obtained using this technique. However, the larger pore might take place from some residual porosity after compression molding process leading to dominant heterogeneous pore size.

2.1.2.5 Fiber Bonding

This technique provides a large surface area to volume ratio giving a desirable as scaffold materials. Mikos *et al.* [39] fabricated PGA scaffolds by fiber bonding technique. PGA fiber was immersed in PLLA solution. After the solvent was evaporated, the PGA fiber was embedded with PLLA leaving an interpenetrating network of PGA and PLLA. The composite was then heated to above the melting temperature of both polymers leading to bond the fibers at their junctions. The PLLA was then dissolved in methylene chloride to give a porous scaffold of PGA. Although this technique could produce interconnected pores, the remaining organic solvent from incompletely removing could be toxic to cells.

Each technique has its advantages, but none can be employed to fabricate for all tissues. Therefore, things to consider depend on the properties of polymer and its intended application.

Up to now, both natural (collagen, gelatin and chitosan) and synthetic (PGA , PLLA and their copolymer PLGA) materials have been studied for use as tissue engineering scaffolds. Since synthetic polymers were lack of cell adhesion, and their surfaces were hydrophobic hindering cell growth in a three-dimensional architecture. Moreover, some polyesters release acidic degradation products which are harmful to host tissue. In addition, the hydrolysis of PLGA copolymers have been shown a significant reducing osteogenesis of healing bone during polymer erosion. Nowadays, many researches have been gave much attention to develop natural polymers as a scaffold instead with outstanding properties, chitosan is one of biopolymers widely used in this field.

2.2 Chitosan

Chitosan, derived from chitin- a natural abundant aminosaccharides, is a copolymer of mostly *N*-glucosamine units and less than 40% *N*-acetyl-glucosamine units. Chitosan can be classified into 3 types according to a crystallization arrangement, α-, β- and γ -forms depending on the sources (Figure 2.1). The different chain arrangement implies different networks of hydrogen-bonds. The α-form, found in shrimp and crab shells, is the most abundant and stable form where the molecules are aligned in an anti-parallel fashion. This type of arrangement gives the most complex network of hydrogen-bonds. While the chains in the β-form chitosan are packed in a parallel pattern, resulting in a weaker intermolecular force and less stable than α-form. The last form, γ -form, is a mixture of α- and β- forms having both parallel and anti-parallel arrangements [40].

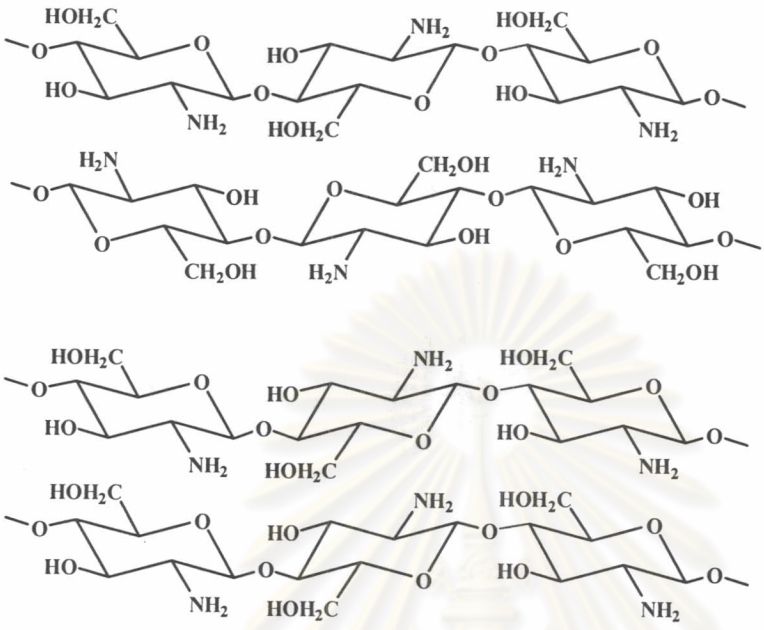


Figure 2.1 Molecular structure of α- and β-form chitosan.

Since chitosan is a renewable material from an exoskeleton of crustacean, cuticle of insects, and cell wall of fungi, numerous researchers have been made an effort to investigate its advantages. Surprisingly, many good properties found in it including biodegradability and biocompatibility. Chitosan is degraded by enzymes and give non-toxic by-products. Others also reported that chitosan are cell-stimulating [13], blood anticoagulability, accelerate wound healing [14], and has hemostatic capability. With these remarkable properties, chitosan has been used increasingly in biomedical application such as a wound healing agent, bandage material, skin grafting template, hemostatic agent, absorbable sutures and drug delivery devices [41]. Despite it cannot be dissolved in common organic solvents, it can be easily processed into various forms, for example, film, powder, gel, bead, fiber, and sponge. Chitosan can be hydrolyzed by chitosanase and lysozyme, an important enzyme in the human body fluid. The degradation leads to cleavage at (1→4) β-linkages of *N*-acetylglycosamine. The *N*-acetylglycosamine moiety in chitosan is a structural feature also found in the glycosaminoglycan, one compound of the ECM affecting cellular movement, shape, proliferation and differentiation [14]. Many usages of chitosan are given in the following.

Biagini *et al.* [14] prepared gel from *N*-carboxybutyl chitosan for use in wound management. The gel facilitated diffusion of substances and cell proliferation. As a result, the wound healing with *N*-carboxybutyl chitosan was better orderly structure and vascularization within 7 days without noticeable scars and inflammatory cells at dermal level.

Mahidally *et al.* [34] reported methods of fabrication chitosan into various devices for tissue engineering, including porous membranes, blocks, tubes, and beads. They suggested to use a cylindrical or cubical scaffold for space filling implants for soft tissue applications, planar scaffolds for skin or articular cartilage, porous beads for cell expansion in bioreactors, and tubular shape for blood vessels. This paper confirmed the easily formation in many shapes of chitosan scaffolds.

Lu *et al.* [42] has demonstrated that the chitosan solution injected into the knee articular cavity of rats caused a significant increase in the density of chondrocyte in the knee articular cartilage, suggesting that the chitosan could be potentially beneficial to the wound healing of articular cartilage.

Haipeng *et al.* [43] reported the use of chitosan and their derived-chitosan as a scaffold for nerve repair. The derived-chitosan studied, glutaraldehyde-crosslinked chitosan, glutaraldehyde-crosslinked chitosan-gelatin conjugate, a chitosan-gelatin mixture, chitosan coated with polylysine (CAP), and a chitosan-polylysine mixture (CPL), were found to be a good material suitable for supporting nerve cell growth.

Prasitsilp *et al.* [44] studied how degree of deacetylation of chitosan from two different sources, shrimp and cuttle fish, affected *in vitro* cellular responses. Without differ from chitosan source, the results indicated that cells more readily attached to higher deacetylated chitosan.

Ma *et al.* [11] fabricated bilayer structure of film and sponge from chitosan imitating the structure of normal skin. The film was prepared by casting method giving the film thickness of 19.6 μm, thereafter a chitosan sponge was formed on film by freezing and lyophilization. Human neofetal dermal fibroblasts were seeded

in the chitosan material remaining stable in shape and size during the cell culture and would be good alternatives to some collagen materials.

Shanmugasundaram *et al.* [45] found that the scaffolds using collagen and chitosan in the form of interpenetrating network (IPN) could be utilized as a substrate to culture Hep-2 cells. It was also used as an *in vitro* model to test anticancerous drugs.

Mi *et al.* [46] developed an silver sulfadiazine (AgSD)-incorporated chitosan membrane by using a dry/wet phase separation method to improve the traditional treating acute burn wounds by using AgSD cream. The bilayer chitosan membrane used as a wound dressing consisted of a dense skin and sponge-like porous layer. The asymmetric chitosan membrane acted as a rate-release controlling wound dressing of AgSD. The bacteria-cultures and cell-culture assay of the AgSD-incorporated chitosan membrane showed the long-term inhibition of the growth of bacteria and decreased potential silver toxicity.

2.3 Photo-Crosslinking

A critical factor for scaffold fabrication design is shape retention. Principally, pore size is an important factor for cell implantation. The pore size should remain somewhat constant during the usage period. Nehrer *et al.* [20] reported that a proper pore size to each cell size gave a greater number of cells. However, biopolymers usually swell very good in physiological fluid, thus have difficulties in retaining the shape of scaffold, as well as the pore size. Furthermore, the scaffold needs to be maintained their stability for surgical handling during implantation. Shanmugasundaram *et al.* [45] reported that chitosan was mechanically weak and quite unstable to maintain a predefined shape for transplantation as a result of swelling.

Crosslinking seems to be the best approach to overcome these problems [47]. As shown in numerous papers up to date, chemical and physical crosslinking have been used to improve the properties of biological biomaterials. Physical crosslinking involves the use of photo-oxidation, dehydrothermal treatments, ultraviolet radiation

[21], and ionic interaction. Overall procedures aim to avoid using toxic chemical agents [25,48,49,50].

For the fabrication of chitosan scaffold, there are reports of crosslinking by both physical and chemical means. Li *et al.* [22] reported methods of using alginate to physically crosslink with chitosan in order to improve mechanical and biological properties for bone tissue engineering. It was found that the compressive modulus of chitosan-alginate scaffold was ~3 times higher than that of pure chitosan, as a result of the strong ionic bonding between amine groups of chitosan and carboxyl groups of alginate. Moreover, osteoblast cells appeared to attach and proliferate well. *In vivo* study showed that the chitosan-alginate scaffold promoted rapid vascularization and deposited connective tissue and calcified matrix within the scaffold.

For chemical treatment, glutaraldehyde is routinely used since it is an inexpensive, water-soluble reagent, making a large number of possible reactions that allow crosslinking. Many works of glutaraldehyde crosslinking found in drug delivery applications [23,24]. However, due to its cytotoxicity showing cell-growth inhibition even at low concentration [25] and potential calcification in applications, many researchers attempt to use other reagents instead.

2.3.1 Azides

Among various crosslinking reagents, azide groups ($-N_3^-$), widely used in biochemistry, are capable of inducing crosslinking which numerous researchers are interested. A number of literatures showed their suitable usages in biomedical application as follows.

Petite *et al.* [51] developed a collagen-crosslinking method by using an azide instead of glutaraldehyde as a crosslinking agent. They reported the incorporation of acyl azide into collagen which carboxyl groups of acyl azide react with free amino groups of collagen. The thermal stability of the collagen increased, as measured by differential scanning calorimetry. The denaturation temperature (T_d) of pericardial collagen treated with acyl azide was raised to 83.4°C. The chemical solubilization

and enzymatic digestion resistance of acyl azide treated tissue was identical to that prepared from glutaraldehyde.

Ono *et al.* [53] presented a photo-crosslinkable chitosan, obtained from chitosan conjugated with *p*-azidebenzoic acid and lactobionic acid (Az-CH-LA) for biological adhesive for soft tissues (Figure 2.2). The lactose moieties was introduced in order to increase water-solubility of chitosan at neutral pH. Az-CH-LA was photo-crosslinked by UV irradiation producing an insoluble hydrogel within 60 s. Biological adhesive test of Az-CH-LA was compared to the fibrin glue. The crosslinked chitosan gel was found to be highly effective in sealing air leakage from pinholes on isolated small intestine and aorta and from incisions on isolated trachea. Furthermore, it showed non-toxic to human skin fibroblasts, coronary endothelial cells, and smooth muscle cells. *In vivo* test, all mice studied survived for at least 1 month after implantation. Therefore, this Az-CH-LA had a potential for serving as a new tissue adhesive.

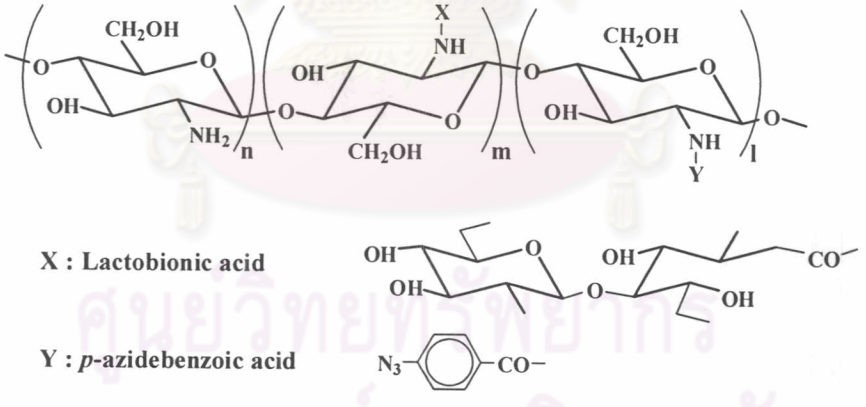


Figure 2.2 Chemical structure of photo-crosslinkable Az-CH-LA.

Jameela *et al.* [23] reported the preparation of photo-crosslinkable chitosan as a drug delivery matrix (Scheme 2.1). A crosslinker, 1-chloro-2-hydroxy-3-azidopropane, was synthesized by the reaction of epichlorohydrin and sodium azide in water. Then it was coupled onto chitosan to obtain photo-crosslinkable chitosan. Drug delivery testing was prepared by casting the films containing drug, such as theophyline. The films were further crosslinked by UV light. IR spectra indicated

complete surface crosslinking within 2 h of irradiation. In drug delivery testing in simulated gastric and intestinal fluids, the release of the drug from the crosslinked films was slower than that from the uncrosslinked films which released completely in a short time. In conclusion, it was possible to use the crosslinked films as drug delivery matrix.

Scheme 2.1 Synthesis of photo-crosslinkable chitosan using 1-chloro-3-azido-2-propanol as a crosslinker.

Zhu *et al.* [53] presented covalent immobilization of chitosan/heparin complex with 4-azidobenzoic acid (Az) as crosslinking agent on polylactic acid (PLA) surface to enhance the lack of mammalian cell adhesion and growth of PLA. Chitosan (CS) was reacted with Az giving Az-CS before it was immobilized on to PLA film surface by UV irradiation. Finally, heparin was modified by. They found that these PLA modifications could inhibit platelet adhesion and activation. Moreover, cell culture (L929 mouse cell) testing showed enhanced cell adhesion.

Obara *et al.* [54] prepared photo-crosslinkable chitosan (Az-CH-LA) hydrogel for wound healing application. The flexible hydrogel was prepared by UV irradiation to Az-CH-LA containing fibroblast growth factor-2 (FGF-2) within 30 s. They found that FGF-2 molecules incorporated into the chitosan hydrogel were gradually released upon biodegradation of the hydrogel itself. Furthermore, FGF-2 incorporated chitosan hydrogel demonstrated a substantial effect to improve wound healing in the mice.

Mao *et al.* [55] used the photosensitive hetero-bifunctional crosslinking reagent, 4-azidobenzoic acid, to attach to *O*-butyrylchitosan (OBCS) for improving the blood compatibility of silicone rubber (SR) film. The modified OBCS was covalently immobilized onto SR film surface before crosslinking by UV irradiation.

The results suggested that the increased blood compatibility of modified OBCS introduced on SR film showed a potential to use as biomaterials.

Dusseault *et al.* [56] developed a method to increase the strong of microcapsules by creating the covalent links between the different layers of microcapsule membrane. *N*-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOS) was used as a crosslinker to react with poly(L-lysine) (PLL) before further incorpolated with cell-containing alginate beads. UVA was used to induce covalent links between the phenyl azide group of PLL-ANB-NOS compound and alginate. It was found that covalently linked microcapsules remained intact after 3 years in a strong alkaline buffer (pH 12), whereas standard microcapsules disappeared within 45 s in the same solution. Moreover, the number of broken standard microcapsules was more than that of covalently crosslinked microcapsules. These results were confirmed the resistance to chemical and mechanical stresses of covalently linked microcapsules.

Masuoka *et al.* [57] presented a photo-crosslinkable chitosan (Az-CH-LA) using *p*-azidebenzoic acid as a crosslinker to serve as a carrier of fibroblast growth factor-2 (FGF-2). The retained FGF-2 molecules in the chitosan hydrogel remained biologically active, and were released from the chitosan hydrogel upon the *in vivo* biodegradation of the hydrogel. Moreover, it was found that the chitosan has a low affinity (K_d) for FGF-2 and prolonged the biological half-life of FGF-2. Furthermore, chitosan was able to protect FGF-2 from heat, acid, and proteolytic inactivation.

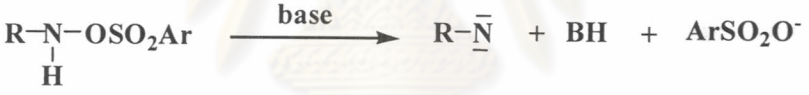
Zhu *et al.* [58] suggested a novel method to modify expanded polytetrafluoroethylene (ePTFE) surface as a vascular graft using chitosan/heparin (CS/Hp) complex. Chitosan was modified with *p*-azidebenzoic acid following coated onto ePTFE surface by UV irradiation. Heparin was further bonded to form complex with chitosan. *In vitro* blood compatibility result exhibited the reduction of platelets adhesion and improving biocompatibility. Corresponding to *in vivo* experiments in dog veins, the unclogged of these grafts was found two-weeks post implantation. From these results, they suggested the use of chitosan/heparin complex on ePTFE dissolving blood clot problem found in small-diameter vascular grafts.

The azide group can be decomposed when exposed to light or UV and gives rise to highly reactive *nitrene* which can react further with several chemical moieties as discussed in the following.

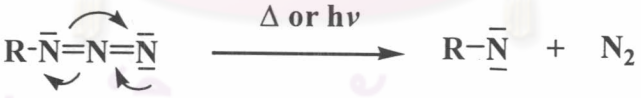
2.3.2 Nitrenes

Nitrene, the nitrogen analogs of carbene, has long been known as intermediates in organic chemistry. Nitrene is a molecular fragment with six electrons on the nitrogen which are formed thermally or photochemically from hydrazoic acid (HN_3) or organic azides. The ground state of nitrene is a singlet with two paired sets of free electrons on the nitrogen, or the triplet with one pair and two electrons of parallel spins [59]. Two principal means to generate nitrenes are:

1) Elimination

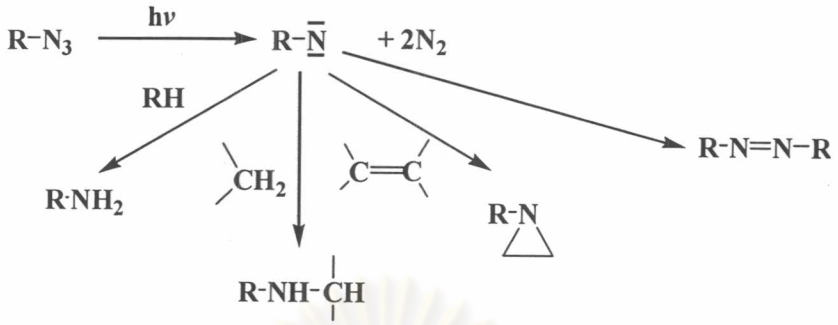


2) Breakdown of certain double-bond compounds from azides



The most common method for generating nitrene intermediates is by thermolysis or photolysis of azides. Many types of azides having been used for generation of nitrenes include alkyl, aryl, acyl, and sulfonyl derivatives [60].

Since nitrenes are too reactive, many reactions in which nitrene intermediates are suspected probably do not involve free nitrenes. Various reactions of nitrenes involve C-H insertion, addition to C=C bonds, rearrangements, abstraction and dimerization as shown in Scheme 2.2.



Scheme 2.2 Photolysis of organic azides and the reaction of nitrene with various organic components.