CHAPTER 6   *PREPPARATION OF PEPTIDE CONJUGATED MICELLE*

6.1 Introduction

Active targeting of micelles to target cells is generally attempted by conjugating targeting ligands to the micelle surface which allow a specific interaction with the target cells. Several type of ligands have been used for this purpose, including antibodies or antibody fragments, vitamins, glycoproteins, peptides (RGD-sequences), and oligonucleotide aptamers. Among the different approaches of active targeting, Peptide Conjugated Micelles using antibody or antibody fragment as a targeting ligand and a lipid vesicle as a carrier for both hydrophilic and hydrophobic drugs, is a fascinating prospect in neuro-therapy. The use of peptide molecule as a homing device has been especially facilitated by the development of the hybridoma technology, which makes it possible to produce a large quantity of a homing peptide to a wide variety of cell determinants (1). However, only a limited number of preclinical studies reported successful targeting of Peptide Conjugated Micelles in vivo (2). Yang ZZ et al have prepared targeted peptide drug conjugates for targeting through nasal administration (3). As systemic administration is the most practical route for the treatment, Peptide Conjugated Micelles must be developed so that physiological barriers can be overcome. For a given a suitable ligand with high specificity and affinity for the target moiety, the critical factor is the accessibility of target cells to Peptide Conjugated Micelles. The process of targeted drug delivery with Peptide Conjugated Micelles can be roughly divided into two phases: the transport phase, in which the Peptide Conjugated Micelles travel from the site of administration (often i.v. administration) to the target cells, and the effector phase that includes the specific binding of Peptide Conjugated Micelles to the target cells and the subsequent delivery of entrapped drugs (2). Finally there are very less reports of application of Peptide Conjugated Micelles for targeting through nasal route.

Peptide Conjugated Micelles for the treatment of cerebral ischemia should satisfy a number of requirements aimed at maximum targeting effect of peptide conjugated nomicelle administered intranasally. Antigen binding site of the micelle-conjugated peptide must be accessible for unperturbed interaction with antigen on the surface of target cells. Peptide conjugated micelles must allow efficient loading and retention of a selected neuroprotective drug. And finally, the drug and peptide incorporation must be stable enough to permit micelle targeting to ischemic tissue without the loss of either of these agents.

The ability to conjugate a homing peptide to drug delivery system is critically important for lots of applications in life science research, diagnostics, and therapeutics. The preparation of ligand conjugates to find and target ischemic cells *in vivo* has become one of the leading strategies of research into investigational new drugs (4). In most cases, the site-specific delivery of drugs involves the successful development of defined ligand conjugates that can target diseased cells without affecting normal cells. The chemistry used to effect conjugate formation should be chosen to yield the best possible retention of antigen binding activity. Required functional groups can be inserted into peptide suitable for modification or conjugation purposes. Crosslinking reagents may be used to target lysine ε-amine and N-terminal α-amine groups. Carboxylate groups also may be coupled to another molecule using the C-terminal end as well as aspartic acid and glutamic acid residues. Although, both amine and carboxylate groups are as abundant in peptide as they are in most proteins, the distribution of them within the three dimensional structure of an immunoglobulin is nearly uniform throughout the surface topology. For this reason, conjugation procedures that utilize these groups will crosslink somewhat randomly to nearly all parts of the peptide molecule. This in turn leads to a random orientation of the peptide within the conjugate structure, often blocking the antigen binding sites against the surface of another coupled protein or molecule. Obscuring the binding sites in this manner results in decreased antigen binding activity in the conjugate compared to that observed for the unconjugated peptide (5).

As discussed before focal cerebral ischemia is caused by a sudden interruption in the blood supply to the brain. Hong et al have attempted to identify peptides that can target to ischemic stroke tissue and detect the apoptosis of cells. After screening of random peptides homing to ischemic stroke tissue in a rat transient middle cerebral artery (MCA) occlusion model they found CLEVSRKNC sequence was most frequently occurring and preferentially homed to ischemic stroke tissue after intravenous administration into the MCA occlusion rats. After intravenous administration CLEVSRKNC peptide concentration increased into an ischemic lesion at the first day and peaked at the third day after the injury. These results demonstrate that the CLEVSRKNC peptide can home to ischemic stroke tissue, while detecting apoptotic neuronal cells, and suggest it has applications as a targeting moiety for molecular imaging and selective drug delivery to stroke tissue. Thus here we hypothesize that the micelles, as a carrier of anti-ischemic drug, conjugated with stroke homing peptide CLEVSRKNC, as a targeting ligand, will carry the drug selectively to the ischemic zone.

Thus, this combined strategy has the potential to overcome some major limitations of conventional anti-ischemic therapy.

Silver Staining:

Silver staining is the most sensitive method for permanent staining of proteins or nucleic acids in polyacrylamide gels. It helps in recording the electrophoresis result that can be viewed without any special equipment. But, the method is very complex with multiple steps and these steps can influence the result. Reagents of high purity and accurate timing will help in achieving reproducible, high-quality results.
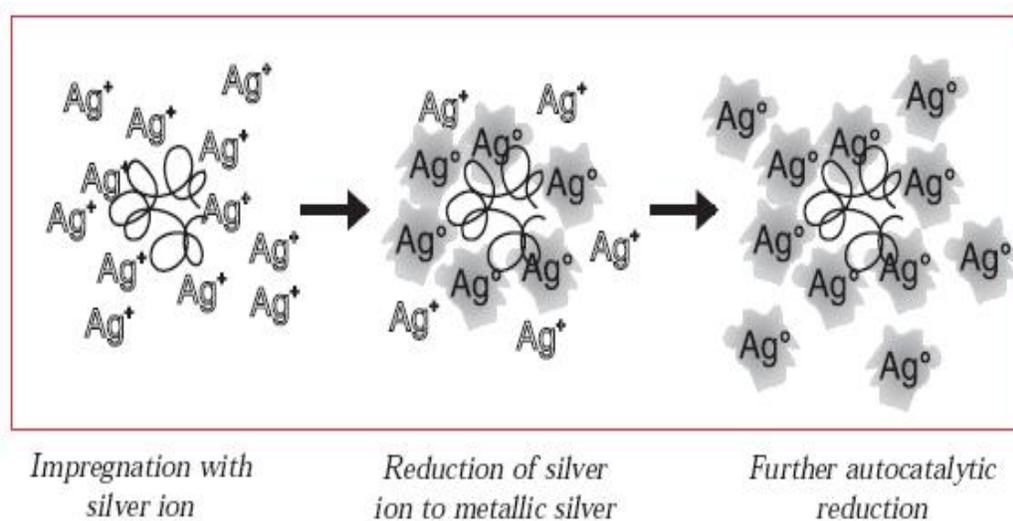

Impregnation with silver ion   Reduction of silver ion to metallic silver   Further autocatalytic reduction

Figure 6.1 Mechanism of silver staining of separated proteins on SDS-PAGE gel.

In silver staining, polyacrylamide gels are saturated with soluble silver ion (Ag^+) and developed by treatment with a reductant. Macromolecules in the gel reduce silver ion to insoluble and visible metallic silver (Ag^o), helps in observation of clear bands containing protein or nucleic acid. The silver staining involves steps such as: Fixing, sensitization, silver impregnation, development, stopping, and gel preservation with in between water wash steps.

Fixing: It involves acid treatment of gel. Acid treatment makes the macromolecules in the gel insoluble and prevents them from diffusing out of the gel during subsequent staining steps. Interfering molecules such as buffers, ions, denaturants, detergents or carrier ampholytes are removed out of the gel during this step.

Sensitization: It involves treatment of gel with reagents that will modify proteins, converting them to a more reactive form toward silver, and reagents that accelerate the subsequent reduction of silver ion. This step is required for enhancing the sensitivity of silver staining of protein but not necessary required for silver staining of DNA. Excess sensitization reagent results in a high level of background staining, so the gel is washed thoroughly with distilled or de-ionized water following the sensitization step.

Silver Impregnation: In this step, the gel is treated with silver nitrate. Mildly acidic conditions prevent silver ion from being reduced to metallic silver. The gel is briefly washed following this step to remove excess silver from the gel surface.

Development: The development solution contains formaldehyde, which reduces silver ion to metallic silver. This reaction only proceeds at high pH, so sodium carbonate is included to render the development solution alkaline.

Stopping and Preservation: The stopping solution prevents further reduction of silver ion. The preservation solution contains glycerol, which prevents the gel from cracking during drying.

6.2 Materials and Methods

CLEVSRKNC peptide was purchased from BD Biosciences, India. Ellman's reagent was purchased from Sigma Aldrich, Mumbai, India. Protein markers were purchased from Fermentas Molecular Biology Tools, Thermo Scientific, US. All other reagents used were of analytical grade and were used without further purification.

6.2.1 Ellman's assay to confirm the presence of –SH groups on CLEVSRKNC peptide

The presence of sulfhydryl groups on the CLEVSRKNC peptide was tested before incubation with functionalised micelles to prepare Peptide Conjugated Micelles using Ellman's reagent. The 30 µl CLEVSRKNC peptide solution was diluted to 500µl with reaction buffer (0.1M sodium phosphate pH 8, containing 1mM EDTA) in a eppendroff. The blank eppendroff containing 500µl reaction buffer was prepared. To both the eppendroff 2.5mL reaction buffer and 50µl Ellman's reagent (4mg dissolved in 1mL reaction buffer) was added, mixed and incubated at room temperature for 15 minutes. The absorbance of the blank ant test samples were taken at 412nm using UV-visible spectrophotometer. The absorbance of test sample

after subtracting the blank absorbance was used for sulfhydryl estimation using molar absorptivity of TNB (2-nitro-5-thiobenzoic acid).

6.2.2 Preparation of Peptide Conjugated Micelles

The CLEVSRKNC peptide with thiol moiety at one end were mixed with the prepared functionalised micelles containing maleimide-terminated linker at the, CLEVSRKNC /Mal-mPEG$_{2000}$-DSPE, molar ratio of 1/100, μM/μM and the mixture was incubated overnight in cold room under N$_2$. Subsequently, the unconjugated maleimide groups were reacted by incubating Peptide Conjugated Micelles with excess amount of cysteine for overnight in cold room. The excess unreacted cysteine was then removed by ultracel membranes (50KDa). The prepared Peptide Conjugated Micelles were confirmed by 8% SDS-PAGE gel permeation chromatography and were analysed for mean particle size, % drug content, and total protein content by Bradford protein estimation method, and for immunoreactivity.

6.2.3 Characterization of Peptide Conjugated Micelle

6.2.3.1 Determination of Sulfhydryl Groups on Peptide

The presence of sulphydryl group was identified using Ellman's reagent and quantified based on molar absorptivity. The amount of thiol groups present in 10μl of the sample containing ~0.834 μg of CLEVSRKNC peptide was calculated below.

The reported molar absorptivity (molar extinction coefficient, which is expressed in units of $M^{-1}cm^{-1}$) of TNB (2-nitro-5-thiobenzoic acid) in this buffer system at 412nm is 14,150 M-1cm-1 (Riddles et al., 1983). Molar absorptivity, 'E' is defined as follows:

$$E = A / bc$$

Where, A = absorbance, b = path length in centimeters, c = concentration in moles/liter (=M)

Solving for concentration gives the following formula:

$$C = A / bE$$

A=0.156, b=1cm and E = 14,150 $M^{-1}cm^{-1}$.

Therefore,   C = 0.156/1(14,150) M

$$= 1.1024 \times 10^{-5} \text{ M/litre}$$

This value represents the concentration of the solution in the spectrophotometric cuvette. To calculate the concentration of the unknown sample, it is necessary to account for dilution factors as follows:

The total volume of the solution being measured is

2.5 ml of Reaction Buffer

+ 0.25ml un-known sample

+ 0.05 ml of Ellman's Reagent Solution

2.80 ml of solution

The concentration of the unknown sample is 1.1024×10^{-5} M (1000ml contains 1.1024×10^{-5} M), then 2.80 ml of that solution contains =

$(2.8 \times 1.1024 \times 10^{-5})/1000 = 3.086 \times 10^{-8}$ moles

Therefore, the 10µl of the used sample (~0.834 µg of CLEVSRKNC peptide) contain 3.086×10^{-8} moles of thiols. This indicated the availability of sulphydryl groups on the CLEVSRKNC peptide for conjugation over micelles through maleimide functional group *via* thioether linkage.

6.2.3.2 SDS-PAGE Analysis of Peptide Conjugated Micelles

The SDS-PAGE gel permeation chromatography was performed to determine the conjugation of CLEVSRKNC peptide to functionalised micelles (Peptide Conjugated Micelles) (6).

6.2.3.2.1 Gel Casting and Electrophoresis

Reagents: 30% Acrylamide Mixture (29.2% Acrylamide + 0.8% bisacrylamide) solution was prepared in milli Q water and stored at 4 ^{0}C in amber coloured bottle. 1.5 M Tris HCl, pH 8.8 (resolving buffer) buffer was prepared in milli Q water and pH was adjusted with concentrated HCl. 1M Tris HCl, pH 6.8 (stacking buffer) buffer was prepared in milli Q water and pH was adjusted with concentrated HCl. 10% SDS solution: 10gm of SDS was weighed and added to milli Q water, heated to 70ºC to dissolve SDS completely, and volume was made up to 100mL with milli Q water. 10% APS (Ammonium persulfate) solution in milli Q water: Stored at -20 ^{0}C. N,N,N',N'-Tetramethylethylenediamine (TEMED). Tank

Buffer: 196mM Glycine, 50mM Tris HCl and 0.1% SDS. Mini gel dual, assembly, Power Pack and Resolving gel Recipe 30ml volume (for 2 mini gels).

Table 6.1 Composition of resolving gel

Components	8%	10%	15%
H_2O	13.9mL	11.9mL	6.9mL
1.5M Tris-HCl, pH 8.8	7.5mL	7.5mL	7.5mL
10% (w/v) SDS	0.3mL	0.3mL	0.3mL
Acrylamide/Bis-acrylamide (30%/0.8%, w/v)	8mL	10mL	15mL
10% (w/v) ammonium persulfate (APS)	0.3mL	0.3mL	0.3mL
TEMED	0.18mL	0.12mL	0.012mL

APS and TEMED were added at the end.

Depending upon the molecular weight of the protein of interest the gel % was chosen.

Table 6.1 Composition of stacking gel

Stacking gel (5%)	5mL
H_2O	3.4mL
1M Tris-HCl, pH 6.8	0.63mL
10% (w/v) SDS	0.05mL
Acrylamide/Bis-acrylamide (30%/0.8%, w/v)	0.83mL
10% (w/v) ammonium persulfate (APS)	0.05mL
TEMED	0.005ml

APS and TEMED were added at the end.

6.2.3.2.2 Sample Loading Buffer Composition

The cell lysates equivalent to 50μg of total protein were mixed with 3X sample loading buffer (**Table 6.3**) in a ratio of 2:1 and then samples were boiled for 5min in a boiling water bath to denaturate proteins and then resolved on SDS-PAGE (8%).**Table 6.2** Sample loading buffer composition

Components	3X (10mL)	6X (10mL)
100% glycerol	3mL	6mL
1M Tris pH 6.8	1.5mL	3Ml
SDS	6g	12g
2-Mercaptoethanol	300μl	600μl
Bromophenol blue	0.05%	0.05%

6.2.3.2.4 Protocol for SDS PAGE

The glass plates were swiped with 70% alcohol and assembled. The bottom and the sides of the glass plates were sealed using 1 % molten agarose. When solidified the resolving gel mixture was poured gently leaving some space for the stacking gel. The gel mixture was overlaid with methanol to give a uniform gel front and kept for polymerization for 20-30 minutes. When polymerization was over the methanol and the unpolymerized gel mixture was removed and washed with milli Q water. The stacking gel mixture was poured over the top of polymerized resolving gel and a 1.5mm comb was inserted. Stacking gel was allowed to polymerize, following which the wells were washed with milliQ water and marked. The assembly cathodic and anodic chamber was filled with Tank buffer and the wells were loaded with the denatured sample. Molecular weight marker was also loaded in the well. Electrophoresis was carried out at 25mA for the stacking and then further run was carried out 30mA. Run was stopped when the dye front reached 1mm above the gel end.

The chromatographic buffers containing 1mM EDTA were used to identify conjugation of peptide to micelle, SDS-PAGE gel permeation chromatography was performed.

6.2.4 Silver Staining Protocol

The separated proteins on 8% and 10% SDS-PPAGE gel were identified using highly sensitive silver staining method. The optimized laboratory protocol was used for staining the proteins.

a. The gel was kept overnight in fixing solution composed of methanol: water: glacial acetic acid (50:40:10, v/v).

b. After overnight incubation in fixing solution the gel was washed with 5% methanol for 10 minutes.

c. Gel was washed twice with distilled water, 5 minutes each.

d. Gel was incubated with 0.02% sodium thiosulphate for 2 minutes.

e. Washed the gel with distilled water 3 times, 3 minutes each.

f. Incubated the gel in 0.2% silver nitrate solution for 30 minutes.

g. Gel was washed with distilled water twice, one minute each.

h. Gel was kept in developing solution (2.19% sodium carbonate, 2mL of 0.02% sodium thiosulphate solution and 125µl formaldehyde solution) until the bands are visualised.

i. When the bands are of desired intensity, gel was removed and placed in a stopping solution (1.4% EDTA). The gels were photographed, and preserved.

6.2.5 Determination of Peptide Concentration in Unknown Solution and Micelle Surface

6.2.5.1 Isolation of Conjugate from Reaction Mixer

Sephadex G-25M column was prepared using 10 ml of glass pipette. The column was hydrated by passing an excess of PBS pH 7 through the column. 1 ml of reaction mixture was placed at the top of column and fractions were (0.5–1.0 ml) as collected. Fractions were analyzed by as per the method developed in analytical chapter. Standard 0.5mg/ml bovine serum albumin (BSA) solution in milliQ water was prepared. 1.25, 2.5, 3.75 and 5µg of BSA from standard solution (2.5µl, 5µl, 7.5µl and 10µl, respectively) were transferred to 96 well plate in triplicate. Volume was made up to 25µl with milliQ water. 25µl water was kept as blank. 25µl of prepared CLEVSRKNC peptide solution and fractions of Peptide Conjugated

Micelles were transferred 96 well plate in triplicate. 100μl of Bradford's reagent was added to all sample and blank wells and absorbance was taken at 595nm using ELISA reader with Soft Max Pro software. Protein concentration was automatically determined by the software, using the BSA standard.

6.3 Results and Discussion

6.3.1 SDS-PAGE Aanalysis of Peptide Conjugated Micelles

In the present research we have conjugated CLEVSRKNC peptide over micelles. The Peptide Conjugated Micelles were identified by SDS-PAGE gel permeation chromatography (6).). 10% and 8%, SDS-PAGE gel was used for identification of CLEVSRKNC peptide. The permeation chromatography was performed under non reducing condition (loading buffer containing no reducing agent) because right end of peptide has –SH group susceptible for reduction when sample was heated in the presence of reducing agent in loading buffer during sample preparation. The chromatographic buffers containing 1mM EDTA were used, as EDTA prevents the ion catalysed oxidation of CLEVSRKNC peptide containing sulfhydryl groups, during experimentation in the chromatographic gel in the presence of buffer.

Silver staining is the most sensitive method for permanent staining of proteins or nucleic acids in polyacrylamide gels as compared to other available methods. We used silver staining method as it is very sensitive method and we can easily detect small amount of peptide. Also, using this method we can easily handle small amount of samples without wasting and when we have less amount of peptide for such projects. The molecular weight of separated proteins are determined using software Alpha ease (Alpha Innotech). Lane 1 is protein marker (3.5, 6.5, 14.3, 20.1, 29, 43 kD), Lane 2 is intact peptide CLEVSRKNC and Lane 3 is peptide conjugated micelle. The gel was incubated in the developing solution as long as possible to obtain all possible bands with minimum background color. The Peptide Conjugated Micelles equivalent to double the amount of intact CLEVSRKNC peptide was loaded in the well.
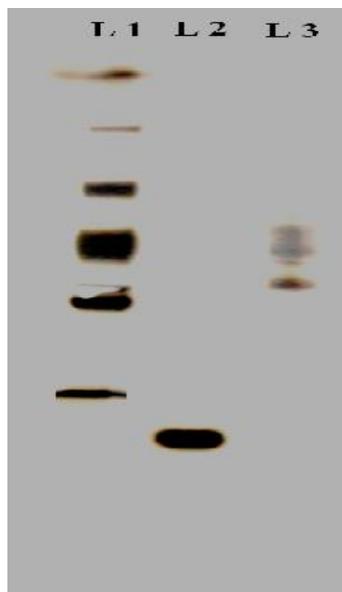
Figure 6.2 Confirmation of CLEVSRKNC peptide and Peptide Conjugated Micelles
(L1: protein marker; L2: intact CLEVSRKNC peptide; L3: Peptide Conjugated Micelles)

The conjugation of CLEVSRKNC peptide over micelles can be confirmed by both the disappearance of intact CLEVSRKNC peptide band and appearance of new band corresponding to Peptide Conjugated Micelles in the lane 3 (**Figure 6.2**). In lane 2, one band is observed at molecular weight of ~1 kDa corresponding to CLEVSRKNC peptide. We observed disappearance of band at ~1 kDa corresponding to CLEVSRKNC peptide and observed very big and well separated new band at ~14.3 kDa indicating the formation of Peptide Conjugated Micelles. The Peptide Conjugated Micelles (composed of many TPGS and PF 127 molecules and CLEVSRKNC peptide) are of high molecular weight, as compared to other proteins used in the study, diffuse very slowly in the gel (8%) and show band near the well, Instead, a new band at ~1.3 kda corresponding to Peptide Conjugated Micelles was observed in our study. The principle of gel permeation chromatography includes diffusion of negatively charged proteins (negative charge induced by heating proteins with SDS during sample preparation) towards positive electrode. The prepared Peptide Conjugated Micelles have zeta potential of -20 ± 3.1mV and they gain still more negative potential during sample preparation for loading.

6.3.2 Preparation of Peptide Conjugated Micelles

The peptide conjugated micelle were prepared by incubating CLEVSRKNC peptide with functionalised PEGylated micelles overnight in cold room followed by incubation with excess amount of cysteine and purification of Peptide Conjugated Micelles from unconjugated CLEVSRKNC peptide fragments and cysteine by using ultracel-50 membranes (50kDa MWCO). **Table 6.4** shows the mean particle size, zeta potential, drug content, and total peptide content of prepared Peptide Conjugated Micelles. The concentration of peptide in unknown solution was determined by Bradford protein assay. To prepare Peptide Conjugated Micelles ~20µg (~0.4µM) of peptide was incubated with functionalised micelles of 0.018mM of total polymer/mL (20 mM of functionalised polymer/mL). The weight ratio of peptide to functionalised lipid is 1:50, w/w (or 1:100, µM/µM). The concentration of peptide attached over micelles was determined by Bradford assay and it was found to be ~14.42±0.12µg/mL (0.0255mM of total lipid). Therefore, the peptide conjugated over micelles was found to be 90.13%.

No change in the zeta potential was observed between functionalized micelle and Peptide Conjugated Micelles.. The % drug content of Peptide Conjugated Micelles was slightly decreased and is might be due to dilution that occur during the preparation and purification of Peptide Conjugated Micelles (**Table 6.4**).

Table 6.3 Comparison Functionalized micelles and Peptide Conjugated Micelles

Formulation	Mean particle size (nm) with PDI	Zeta potential (mV)	% Drug content
PEGylated Micelles	19±2 (0.288±0.050)	-16±3.1	74.42±3.62
Peptide Conjugated Micelles	20±3 (0.265±0.052)	-15±2.6	73.23±3.54

Values are Mean ± SD, n=3.

6.4 Reference

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