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BIOCHEMICAL ASSAY ON INTERACTION BETWEEN HIGH MOLECULAR WEIGHT (HMWC), AND LOW MOLECULAR WEIGHT (LMWC) POLYMERS WITH PLASMID DNA.

Almas kanwal[1], M. Zeeshan Danish[2], Gul Muhammad[1], Ghulam Razaque[1], Nisar A. Shahwani[1], Ghulam Murtaza Qureshi[3]

[1]Faculty of Pharmacy & Health Sciences University of Balochistan, Quetta.
[2]College of Pharmacy, Punjab University Lahore.
[3]Peoples university of medical and health sciences SBA , Nawab Shah Sind.

Abstract:

Polymer based non-viral gene delivery systems depend on the electrostatic interaction between cationic polymers and nucleic acids which yields inter-polyelectrolyte complexes (ipec). Such behaviour of interaction between polycations (cationic polymers) and polyanions (dna/sirna) can be well explained well by polyelectrolyte theory. Further addition of polycations, portion of negatively charged n-pec decreases until over all concentration of polycations and polyanions become equal and all dna/sirna charges are neutralized. Therefore when the portion of the insoluble complex (s-pec) grows the phase separation of complex species takes place and complex solubility decreases. High and low molecule chitosan were selected for this work. Physicochemical characterization of polyplexes formed due to the interaction of high molecular weight (hmwc) and low molecular weight (lmwc) chitosan with plasmid dna and sirna were performed. Gel retardation assay of polyplexes formed due to the interaction of high molecular weight (hmwc) with plasmid dna were done. Gel retardation assay of polyplexes formed due to the interaction of low molecular weight (lmwc) with plasmid dna were also performed. In (hmwc) gel image lane 1 contains polymer only and lane 10 contains plasmid dna only as controls while lane 2-9 contains complexes of polymer and dna with polymer to dna ratios of (0.25:1, 0.5:1, 0.75:1, 1.5:1, 3:1, 6:1, 8:1, and 10:1) respectively. In (lmwc) gel image lane 1 contains polymer only and lane 10 contains plasmid dna only as controls while lane 2-9 contains complexes of polymer and dna (w/w) with polymer to dna ratios of (0.25:1, 0.5:1, 0.75:1, 1.5:1, 3:1, 6:1, 8:1, and 10:1) respectively

Corresponding author:

Almas kanwal,
Faculty of Pharmacy & Health Sciences,
University of Balochistan,
Quetta.

QR code

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INTRODUCTION:

Polymer based Non-viral gene delivery systems depend on the electrostatic interaction between cationic polymers and nucleic acids which yields inter-polyelectrolyte complexes (IPEC) [1]. Formation and solution behaviour of IPEC is just like to that of synthetic polyions produced complexes. Such behaviour of interaction between polycations (cationic polymers) and polyanions (DNA/siRNA) can be well explained well by polyelectrolyte theory [2].

Based on this concept, when nucleic acids (as polyanions) and cationic polymers (as polycations) are mixed with each other in a solution, resulting complexes (IPEC) are stabilized by a system of cooperative salt bonds between the polyanion and polycation repeating units (RU:Nt ratios). Thus, stability of IPEC system depends on the number of salt bonds (ratios of polycation units to phosphate bases of siRNA or DNA molecules) of interacting polyions due to their coupling reaction. The hydrophobic site formed when the polycation binds to the nucleic acid and neutralized the charge present on the phosphate group [3]. The number of these sites are identified by the polynucleotide and polycation or (RU:Nt) units which can also be referred as the "complex composition" (Φ). Initially, at low concentrations of polycations, negatively charged water-soluble, non- stoichiometric complexes (N-PEC) are formed where polycation chains are consistently distributed among the backbone of nucleic acid. By increasing the amount of the polycations the segments of the hydrophobic sites also increased but the complex negative charge decreases and at some position a critical concentration is reached ($\varphi = \varphi c$). Until the polycation binding causes the complexes precipitation there is increase in hydrophobicity of complex. Such change in balance of charges and solution instability results disproportionation [4]. Under such critical conditions irregular division of the polycation chains between the DNA molecules becomes thermodynamically constructive. This leads to the formation of two types of the complexes present in the mixture simultaneously: (1) insoluble stoichiometric polyelectrolyte complexes (S-PEC) i.e charge on the polycation and phosphate groups are balanced i.e $\varphi = 1$ and (2) non-stoichiometric polyelectrolyte complexes (N-PEC) remains in solution with a critical composition ($\varphi < \varphi c$) [5].

Further addition of polycations, portion of negatively charged N-PEC decreases until over all concentration of polycations and polyanions become equal and all DNA/siRNA charges are neutralized [5]. Therefore when the portion of the insoluble complex (S-PEC) grows the phase separation of complex species takes place and complex solubility decreases. By the gradual addition of polycations only the stoichiometric (S-PEC) will be present in the system at a stage when the base molar concentrations of DNA/siRNA and the polycation will become again equal. Further addition of the polycation may lead to the conversion of S-PEC back to N-PEC [6, 7].

Under these conditions due to recharging of the complexes and increase in its solubilization φ becomes = 1, where complex species are stabilized in solution by the excess of positively charged polycation chains linked to polyanion [8]. S-PEC are coacervate but are always in equilibrium with N-PEC in solution and slow exchange of polyelectrolyte molecules takes place between coacervate and solution to maintain between the charged groups in the coacervate. The interaction of polyelectrolytes depends on the relative size or chain length of either component. Largest polyelectrolyte is considered as "Host" polyelectrolyte and the smaller as "guest", thus initial excess of host polyelectrolyte will first favour the formation of soluble N-PEC. Since, excess of either DNA or polycation make IPEC soluble, so there is formation of non-stoichiometric complexes (N-PEC) takes place, which are either positively charged (polycation excess) or negatively charged (DNA excess). When there is formation of stoichiometric complexes (water insoluble) there is a range of the ratio of the polycation and DNA [3]. In the eukaryotic cells number of plasmid DNA are inactive due the negatively charged IPEC. The stoichiometric IPEC cannot be utilized in pharmaceutical preparations due to precipitation. In the excess of the polycations the cationic IPEC are produced, cationic IPEC are both stable in solution and transfect the cells efficiently either by receptor mediated endocytosis or electrostatic interaction. However, the bio-distribution properties and pharmacokinetic of polymer based IPEC should depend strongly on the interactions of the cationic groups which also may impose serious limitations on application of those IPEC in gene delivery [9].

Strength of polyelectrolytes also depends on many factors other factors such as pKa value, hydrophobicity, structure and conformation of polycations. However, polyelectrolyte theory provides the basic information about the electrostatic interaction between nucleic acids and cationic polymers for suitable complex formation. Some of the cationic polymers forming IPEC with siRNA are discussed below [6]. Overall polymer based polyelectrolyte complexes (PECs) for siRNA delivery are considered very promising [10] but suffers from many of the same obstacles as of DNA delivery. Some of these common issues for siRNA

and DNA interactions with polycations include their binding ability, extra and intracellular stability, cellular uptake, endosomal release functionality, and release from its vector into target cells [11, 12]. However, plasmid DNA forms smaller size polyelectrolyte complexes with cationic polymers which protect pDNA against enzymatic degradation and promote uptake into cells via endocytic pathways. In comparison siRNA is believed to be more difficult to condense into small size complexes due to its short chain length [13, 14]. Therefore, effective binding of siRNA with cationic agents becomes a challenge essentially required for its in vivo delivery [15]. A range of DNA delivery vectors including linear [16], and branched [17] polymers, dendrimers [18, 19], chitosans [20], and peptides [21] have already been explored for siRNA delivery as polyelectrolyte complexes (PECS). Although, years of research for DNA gene delivery based on polymeric carriers have revealed valuable evidences for the formation of PECs with some desirable characteristics but the fundamental aspects of siRNA interaction with polycations are still not very clear [22]. Hence, there is still a potential need to understand the underlying reasons that why siRNA is different from DNA in terms of complexation and binding behavior with polycations in forming polyelectrolyte complexes. This gap can be filled by addressing the comparative differences between physicochemical characteristics of siRNA and different DNA molecules in terms of their binding and complexation behavior using polycations of various architect and structure.

MATERIALS AND METHODS:

POLYMERS, CHEMICALS, SOLVENTS, AND OTHER REAGENTS

High and low molecular-weight chitosan, Acetic acid, agarose powder, anhydrous N,N-Dimethyl formamide(DMF),anhydrous tetra hydrofuran(THF),boric acid and cystein hydrochloride, sodium hydride(NaH), sodium hydroxide, sodium tripolyphosphate (TPP), thiobutyric acid, thioglycolic acid and thionyl chloride (SoCl2).Most of the chemicals, solvents and reagents used in this project would be of molecular biological (MB) grade and would be nuclease and RNAase free.

2.4 Buffers and Reagents

The gel retardation assay utilized molecular biology (MB) grade agarose. Ethidium bromide solution (10mg/ml) was purchased as a pre-prepared solution from Sigma Aldrich . Ethidium bromide is carcinogenic and was handled with great care according to the manufacturer's specifications. Tris acetate-EDTA (TAE) stock solution consisted of a solution of 2M Trizma base (tris-hydroxymethyl-aminomethane), 1M glacial acetic acid and 50mM ethylenediamine tetracetic acid, sodium salt dihydrate (EDTA). TEA buffer working solution was made up by 50-fold dilution and then adjusted to pH 7.4 with glacial acetic acid. DNA loading buffer was a solution of 0.25% w/v bromophenol blue and xylenecyanol with 50% glycerol. For Coommassie blue staining and destaining, two solutions were used. The staining solution was the mixture of 0.1% w/v Coommassie blue from Sigma (UK), 50% methanol and 10% glacial acetic acid in water. The destaining solution was a mixture of 10% v/v methanol and 10% v/v glacial acetic acid in water. Phosphate buffered saline (PBS) was prepared from tablets. Reduced ionic strength medium containing 14 mM NaCl and 1 mM phosphate was prepared by a tenfold dilution of PBS in water and then adjusted to pH 7.4.

2.5 Physical characterization

Methods employed in this section to investigate polymer and DNA interaction, resulting in DNA self-assembly into PECs for gene delivery, were categorised into two groups. The first group was those used to assess the nature of polymer–DNA binding that included fluorimetric analysis of the displacement of the dye ethidium bromide (EtBr) from DNA, called EtBr displacement assay, and electrophoretic retardation of DNA by the treatment of a polymer, called gel retardation assay. The second group of methods were those used to characterize the colloidal properties of the PEC complexes resulting from the polymer–DNA interactions. This group comprised of visualisation of complex morphology by transmission electron microscopy (TEM), and size analysis in aqueous environment by photon correlation spectroscopy (PCS) that also provided information regarding the complex solubility and polydispersity. A more detailed understanding of the physicochemical properties of these polymer-DNA complexes was obtained from the following methods.

RESULTS AND DISCUSSION:

3.1. Physicochemical characterization of polyplexes formed due to the interaction of High molecular weight (HMWC) and Low molecular weight (LMWC) Chitosans with plasmid DNA and siRNA.

Complexation and binding ability of nucleic acids with cationic polymers are preliminary and integral features for better transfection and silencing effects both on in vitro and in vivo levels. Therefore, we have initiated our work to start with the basic concept of gel retardation assay and dynamic light scattering techniques. For physicochemical characterization of the complexes formed due to the electrostatic

interaction between polycationic cationic polymers and nucleic acids. This project focused to assess the physicochemical nature of the polyplexes of plasmid DNA and small interfering RNA (siRNA) using difrerent chitosans polymers including High molecular weight (HMWC) and Low molecular weight (LMWC) Chitosans. Polyplexes formed due to the electrostatic interactions of nucleic acid and chitosans polymers were analysed for their comparative complexation and binding behaviour by electrophoresis gel retardation assay and sizes of the polyplexes for both nucleic acids were further investigated by dynamic light scattering (DLS).

However, size and morphology of the nucleic acids were analysed by transmission electron microscopy (TEM).

3.1.1 Gel retardation assay of polyplexes formed due to the interaction of High molecular weight (HMWC) with plasmid DNA.

Gel electrophoresis has been used to determine the complexation behaviour of the high molecular weight chitosand (HMWC) which have been highlighted with upper images and DNA with lower images as clear bands towards respective electrodes. Such behavior of the polycations (Chitosans polymers) provides information about complexation behavior and binding ability due to the interaction of polymer and nucleic acids on gel. The pattern of the binding ability for the polymers and nucleic acid interactions was assessed by using UV trans-illuminator. Two comparator nucleic acids samples plasmid DNA (60kbp) and small interfering (siRNA-21bp) in were used in this project to analyze the comparative binding behavior of DNA and RNA.

Figure 3.1 shows that For High Molecular weight chitosan (HMWC) plasmid DNA has shown no complete complexation till lane 4. However, at lane 5 to 9 all the DNA was complexed fully with chitosans polymer. In figure 3.1 lane 1 indicates the naked polymer and lane 10 contains naked plasmid DNA which has been used as controls. However, lane while lane 2 to 9 contains complexes of polymer and DNA with weight to weight (W/W) polymer to DNA ratios of (0.25:1, 0.5:1, 0.75:1, 1.5:1, 3:1, 6:1, 8:1, and 10:1 respectively.

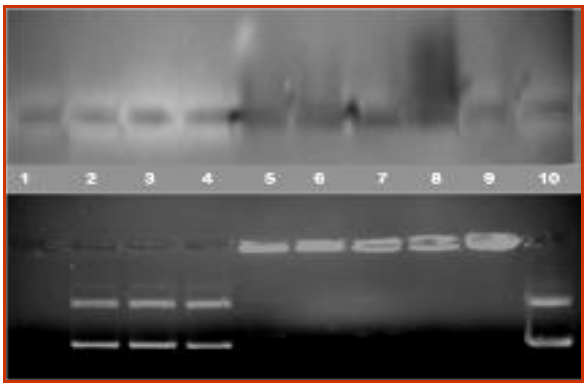

Figure: 3.1. Gel shift assay of the complexes formed at different ratios between plasmid DNA and HMWC in TAE buffer, pH 7.6. The 1.% agarose gel (containing ethidium bromide) was electrophoresed at 80V for one hour. The samples were loaded into central wells on the gel. Free DNA was detected by UV fluorescence. Free polymer (PAA) was detected by staining with comassie blue. In gel image Lane 1 contains polymer only and lane 10 contains Plasmid DNA only as controls while lane 2-9 contains complexes of polymer and DNA with polymer to DNA ratios of (0.25:1, 0.5:1, 0.75:1, 1.5:1, 3:1, 6:1, 8:1, and 10:1) respectively.

3.1.2 Gel retardation assay of polyplexes formed due to the interaction of Low molecular weight (LMWC) with plasmid DNA.

Low Molecular weight chitosan (HMWC) plasmid DNA has shown similar complexation trend where no complete complexation was achieved till lane 4. However, at lane 5 to 9 all the DNA was complexed fully with low molecular weight chitosans polymer. In figure 3.2 lane 1 indicates the naked polymer and lane 10 contains naked plasmid DNA which has been used as controls. However, lane while lane 2 to 9 contains complexes of polymer and DNA with weight to weight (W/W) polymer to DNA ratios of (0.25:1, 0.5:1, 0.75:1, 1.5:1, 3:1, 6:1, 8:1, and 10:1 respectively. Lane 8 and Lane 9 show the dark bands moving away from wells on the opposite side which shows the presence of access polymer.

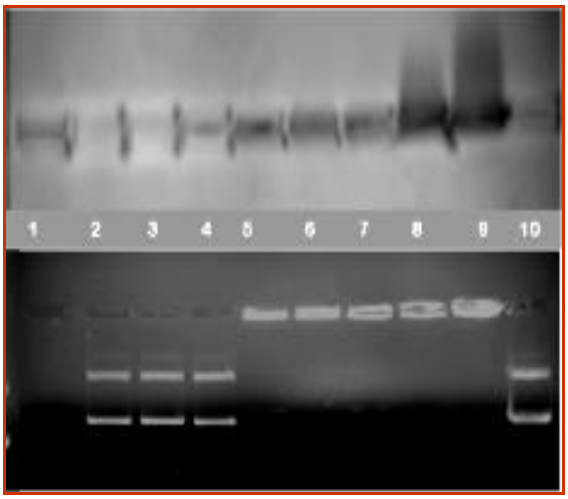

Figure: 3.2. Gel shift assay of the complexes formed at different ratios between plasmid DNA and LMWC in TAE buffer, pH 7.6. The 1 % agarose gel (containing ethidium bromide) was electrophoresed at 80V for one hour. The samples were loaded into central wells on the gel. Free DNA was detected by UV fluorescence. Free polymer was detected by staining with comassie blue. In gel image Lane 1 contains polymer only and lane 10 contains Plasmid DNA only as controls while lane 2-9 contains complexes of polymer and DNA (w/w) with polymer to DNA ratios of (0.25:1, 0.5:1, 0.75:1, 1.5:1, 3:1, 6:1, 8:1, and 10:1) respectively.

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