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Review Article

# Tumor Biology; Usefulness of Thermosensitive and pH Sensitive Polymeric Nanoparticles for Tumor Targeting: A Review

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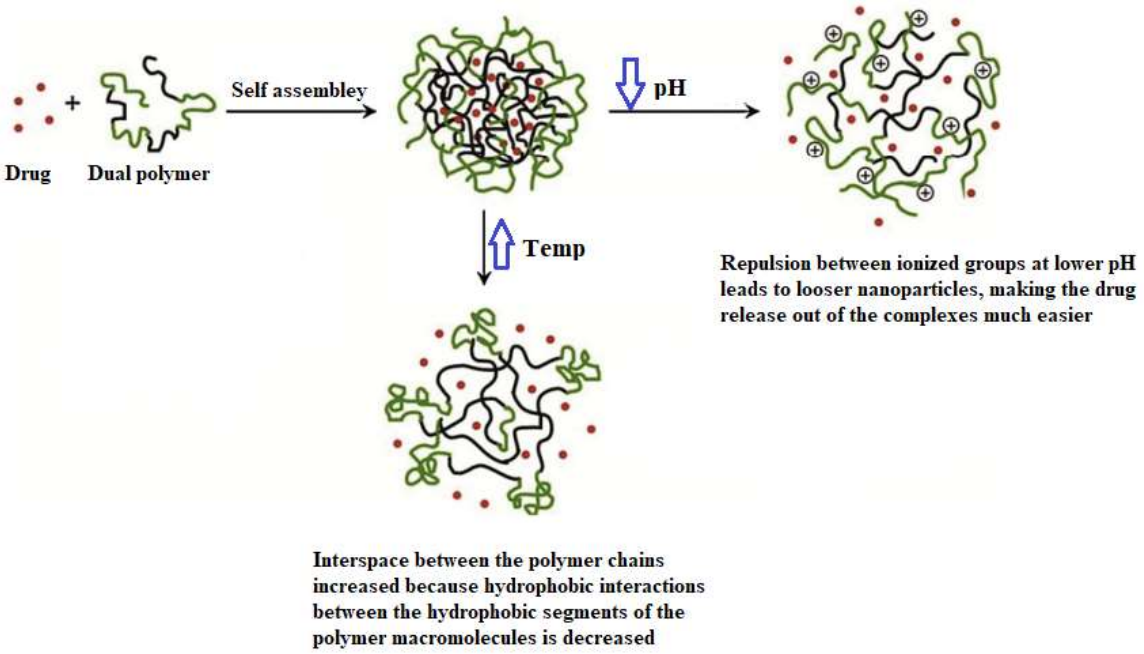
## Abstract

Polymeric nanoparticles (PNPs) have attracted the interest of many scientists and have been utilized in an increasing number of fields during the last two decades. The conventional chemotherapeutic agents have poor pharmacokinetic parameters such as non-specific distribution of drugs, the lack of drug specific affinity towards a pathological site, and thus necessitating large dose of a drug to achieve high local concentration in the body leading to systemic toxicity associated with serious side effects. A thorough and precise understanding of tumor microenvironment such as angiogenesis, tumor pH, enhanced permeation and retention (EPR) effect, abnormal lymphatics, multidrug resistance (MDR) and high interstitial fluid pressure, allows designing drug delivery systems that specifically target anti-cancer drugs to tumors. An attempt has been made in this article to highlight the temperature and pH sensitive PNPs, and PNPs with dual and double response to both temperature and pH that have a distinct capacity to target tumors with limited effect on healthy tissues. The stimuli responsive nanoparticles are classified based on their mechanism of response to temperature and pH. Structure of the polymer, methods of drug loading, and characterization of PNPs are elucidated.

**Keywords:** Polymeric nanoparticles; pH sensitive polymers; Thermosensitive polymers; Tumor microenvironment; Multidrug resistance; Drug loading.

## Graphical Abstract



## 1. Introduction:

Cancer is a disease in which control of growth is lost in one or more cells by multiple changes in gene expression leading to an imbalance between cell division and cell death, thus forming a tumor. The primary tumor often becomes life-threatening by obstructing vessels and organs. However, death is most commonly caused by the spread of the primary tumor to one or more other sites in the body by a process called metastasis, which makes surgical intervention impossible[1]. The treatment given for cancer is variable and dependent on a number of factors including the type, location, stage of cancer, and the health status of the patient. Most treatments are designed to either directly kill or remove the cancer cells or lead to their eventual death by depriving them of signals needed for cell division. Other treatments work by stimulating the body's defences against the cancer cells[2].

Commonly used cancer chemotherapy has presented unsatisfactory results, as the therapy is deleterious to patient health by making them more susceptible to other diseases and often causes death by weakening the patient's immune system. The life-threatening side effects caused by non-specific tissue distribution of anti-cancer agents have restricted the systemic high dose strategy[3]. Cancer cells except those having intrinsic resistance are sensitive to chemotherapy in the beginning; but often develop acquired resistance upon repeated cycles of chemotherapy. The resistance initiated by an anti-cancer agent extends cross-resistance to a wide range of drugs having different chemical structures & cellular targets. Once the resistance develops, systemic high dose administration of anti-cancer agents becomes ineffective, and resistance is further stimulated. Thus, there is a need for developing effective drug delivery mechanisms; PNPs provide an effective alternative to the conventional chemotherapies.

PNPs offer an advantage over other delivery systems in targeting the chemotherapeutic agent to the site of action, along with the reduced delocalization of drug to other sites in the body[4,5]. Stimuli responsiveness of the polymers can be further utilized in drug delivery to tumor, due to precise release of the entrapped active ingredient in response to a particular stimulus such as temperature and pH. In the subsequent part of this review, the chemical modification of the polymers to bring about stimuli responsiveness, drug loading into the nanoparticles, and characterization methods are discussed.

## 2. Exploring the tumor microenvironment:

Numerous physiological barriers in the tumor microenvironment hinder the efficacy of anticancer agents. The drug delivery in the tumor is challenged by chaotic blood supply, poor tumor vasculature permeability, high interstitial pressure, and absence of lymphatic network. The peculiar tumor microenvironment, including tumor angiogenesis, can be exploited to design drug delivery systems that can target tumor [6,7] . Conventional chemotherapeutic agents can trigger serious side effects due to poor pharmacokinetic profiles and non-specific distribution in the body. The knowledge and the precise understanding of the tumor microenvironment allows researchers to elaborate different therapeutic strategies, based on differences with healthy tissues[8].

**2.1. Angiogenesis:** For a solid non-angiogenic tumor (~1-2mm$^3$), oxygen and nutrients can reach the centre of the tumor by simple diffusion. Because of their non-functional or non-existent vasculature, non-angiogenic tumors are highly dependent on their microenvironment for oxygen and supply of nutrients[9].

When tumors reach a size of 2mm$^2$, a state of cellular hypoxia begins, initiating angiogenesis (Fig 1). Activated endothelial cells express the dimeric transmembrane integrin, $\alpha_v\beta_3$, which interact with Extracellular Matrix Proteins (ECM) (vibronectin, fibronectin) and regulate the migration of endothelial cell through ECM vessel formation[10]. The activated endothelial cells synthesize proteolytic enzymes (ex: matrix metalloproteinases) and degrade the ECM and basement membrane. The inner layer of the endothelial cells undergoes apoptosis leading to the formation of vessel lumen. Immature vasculature undergoes extensive remodelling during which the vessels are stabilized by pericytes and smooth muscle cells. This step is incomplete resulting in irregular shaped, dilated and tortuous tumor blood vessels[11].

**2.2. Tumor pH:** While the extracellular pH ($pH_e$) in normal tissues and blood is around 7.4, the extracellular pH in most tumors is more acidic (pH<7.0) than in normal tissues[12]. Although there is a distribution range of 5.7 to 7.8 of *in vivo* pHe among human patients with a variety of solid tumors (adenocarcinoma, squamous cell carcinoma, soft tissues sarcoma, and malignant melanoma) depending on the tumor's histology and volume, the average pH is around 7.0[13]. The intracellular pH ($pH_i$) of normal cells is around 7.2, while the tumor cell cytosolic pH is approximately 7.4[14].

The extracellular acidity of tumor microenvironments is caused in part by lactic acid accumulation in rapidly growing tumor cells owing to their elevated rates of glucose uptake but reduced rates of oxidative phosphorylation[15]. This persistence of high lactate production by tumors in the presence of oxygen is termed as "Warburg's effect" and it provides a growth advantage for tumor cells *in vivo* [16]. Hypoxia induced expression of carbonic anhydrase (IX) will also contribute to exacerbate the pH gradient between the intra and extracellular compartments through the conversion of $CO_2$ to $HCO_3^-$ and subsequent uptake of this weak base through the anion exchange[17].

The resulting pH gradient between intra and extra-cellular tumor compartment, and between the tumor mass and healthy tissue are therefore potential sources of differential drug partitioning and distribution. In a low pH extracellular microenvironment, the uncharged fraction of a weak acid increases and such a drug can thus more easily diffuse through the cell membrane. The relatively basic intracellular compartment may in turn favour the ionization of the molecule, thereby promoting the cytosolic accumulation of the molecule[18].

**2.3. Hypoxia:** Hypoxia refers to a condition in which the tissues are deprived of oxygen, or the partial pressure of oxygen falls below critical levels leading to hindered functioning of cells or organs[19]. In normal tissues, the required amount of oxygen for metabolism is supplied through blood. In contrast, oxygen demand far exceeds supply in tumors as they grow. High cell density caused by excessive rates of cell proliferation in tumors places a huge demand on local supply of oxygen. Furthermore, abnormal tumor vasculature reduces blood flow and limits delivery of oxygen throughout the tumor resulting in regions of hypoxia.

Hypoxia contributes directly to the factors that favour malignant tumor progression through the effect of activity and expression of tumor suppressor proteins such as p53[20,21]. Another major consequence to hypoxia is resistance to chemotherapy due to over expression of ATP-Binding Cassette (ABC) transporters which pump anticancer drugs out of cancer cells[22] and resistance to radiotherapy by making the cells insensitive to radiations[23-26].

**2.4. Enhanced Permeation and Retention effect (EPR):** Tumor blood vessels are characterized by abnormalities such as high proportion of proliferating endothelial cells, pericyte deficiency, and aberrant basement

membrane formation leading to enhanced vascular permeability[7].

Particles, such as nanocarriers can extravasate and accumulate inside the interstitial space. The endothelial pores in tumor tissue have sizes ranging from 10-1000nm. Moreover, lymphatic vessels are absent or non-functional in tumor which contributes to inefficient drainage from tumor tissue. Nanoparticles entered in to the tumor are not removed efficiently and are thus retained in tumor[27-29].

**2.5. Abnormal lymphatics:** The lymphatic network transports interstitial fluid and immune cells out of normal tissue and is essential for immune function and maintenance of fluid balance in tissue interstitium[30]. In contrast, the lymph vessels in tumors are compressed by solid stresses induced by continuously multiplying cells within the microenvironment[31].

Lymphatic vessels at the periphery of the tumor or the periphery- tumor interface function normally, while those with in the tumor are functionally defective[32,33]. Abnormal lymphatics encourage retrograde flow in lymphatic vessels, allow tumor cells to invade the periphery of lymphatic vessels and promote metastasis within the lymphatic system[34,35].

**2.6. High interstitial fluid pressure:** The interstitium of tumor is composed predominantly of collagen and elastin fibers forming a cross-linked network. Interspersed among the fibres are the interstitial fluid and the macromolecules such as hyaluronates and proteoglycans, which together form a hydrophilic gel. The tumor is characterised by high interstitial fluid pressure, forcing the interstitial fluid out of the tumor tissue by a convective flow. Thus, the transport of anti-cancer agent across interstitium is governed by the pressure of the interstitial fluid, its structure, charge, composition, and also by the physicochemical properties of the drug molecule[36,37].

**2.7. Multi Drug Resistance:** The resistance that neoplastic cells manifest to anti-cancer agents may develop either at first exposure to the drug or at subsequent exposure (acquired) [38,81-83]. Acquired resistance may result from adaption of tumor cells through mutations, resulting in the emergence of cells with less susceptibility or increased resistance to the drug.

Various mechanisms (Fig 2) of resistance include[39],

a) Decreased accumulation of cytotoxic drugs in cells due to increased expression of cell surface, energy dependent drug transport proteins, resulting in multidrug resistance to many structurally dissimilar anti-cancer drugs. An important member of this group is P-glycoprotein/MDR1(Table 1) [40].

b) A decrease in the amount of uptake of drug.

c) Insufficient metabolic activation of certain drugs such as Mercaptopurine, Cytarabine, Fluorouracil.

d) Increased inactivation of drugs like Cytarabine, Mercaptopurine.

e) Increased concentration of metabolizing enzyme for drugs like Methotrexate.

f) Increased utilization of alternative metabolic pathways.

g) Rapid repair of drug induced lesions (ex: alkylating agents).

h) Altered activity of target, ex: topoisomerase II (doxorubicin).

i) Mutations in various genes, giving rise to resistant target molecules. For example, the p53 gene and overexpression of Bcl-2 gene family (several cytotoxic drugs).

Thus, to deliver anticancer agents to the tumor tissues *in vivo*, one must overcome (i) drug resistance at the tissue level (physiological barriers) (ii) drug resistance at the cellular level (Multi Drug Resistance) and (iii) distribution, biotransformation, and clearance of chemotherapeutic agents in the body.

Nanoparticles, because of their smaller size, can extravasate through the fenestrations of the tumor blood vessels, and are retained in the interstitium due to the abnormal lymphatic system. The specific characteristic of tumor tissue, such as lower pH and higher temperature can be exploited in targeting drugs through a delivery system which respond to these stimuli[41,42].

## 3. Drug Targeting:

Drug targeting was considered as a hypothetical 'magical bullet' by Pau Ehrlich almost a century ago. The success of this approach depends on two components – 1. Recognising/responding/directing/binding to the target (or) its environment and 2. Eliciting the therapeutic effect [43]. The concept of drug targeting involves co-ordinating behaviour of three components (a) Drug (b) Targeting moiety and (c) Pharmaceutical carrier.

The three major strategies of drug targeting include (i) direct application of drug to the affected area (ii) passive targeting and (iii) active targeting. The successful example of direct application includes the intra-articular administration of hormonal drugs in the therapy of arthritis or intracoronary infusion of thrombolytic enzymes in the therapy of thrombus-induced myocardial infarction[43]. However, the applicability of such a straight approach is limited. Passive targeting utilizes the natural course of bio-distribution of the carrier system, through which, it eventually accumulates in the organ compartments of the body. The EPR effect is the guiding principle which leads to selective accumulation of nanocarriers and drugs in the tumor interstitium [44]. However, passive targeting depends on the degree of tumor vascularisation and angiogenesis. Thus, extravasation of nanocarriers will vary with tumor types and anatomical sizes and the high interstitial fluid pressure of solid tumors avoids successful uptake and homogeneous distribution of drugs in the tumor[45,46].

Active targeting facilitates binding of the drug carrier to the target cells through the use of ligand or engineered homing devices to increase receptor-mediated localization of the drug or chemical modification of the carrier to respond to micro-environmental conditions of tumor and target specific delivery of drug(s)[47]. Stimuli sensitive PNPs can be utilized in active targeting by both ligand mediated and physical targeting strategies[46].

## 4. PNPs

The physicochemical properties of polymers that greatly influence the properties, method of preparation, and the performance of the nanocarrier include the molecular weight, degree of crystallinity, hydrophobicity, copolymer ratio, biodegradability, biocompatibility, solubility, and drug-polymer interactions[48].

### 4.1. Structure of the polymers forming nanoparticles:

Polymeric micelles are composed of amphiphilic block copolymers. The hydrophobic blocks constitute the core of a polymeric micelle. A research group lead by H. Ringsdorf, in 1984, pioneered their use as a drug delivery tool[49]. Subsequently in early 1990's, doxorubicin-conjugated block copolymer micelles were developed. An interest in the

polymeric micelles as drug delivery tools is on the rise in the past few decades[50].

The amphiphilic block copolymers generally consist of a hydrophilic block and a hydrophobic block. When the concentration of the block copolymers in an aqueous solution increase above a certain concentration, called the "Critical Micellar Concentration (CMC)" or "Critical aggregation Concentration (CAC)", they form micelles. At CMC or CAC, the hydrophobic segments of the block copolymers start to associate by hydrophobic interactions, although other interactions such as electrostatic interactions and sterio-complex form to reduce the area of contact with water resulting in the formation of vesicles or core-shell micellar structures[51]. The hydrophilic blocks of the copolymers form the shell of the micelle and stabilize the system.

Polyethylene glycol (PEG) is the most commonly used hydrophilic segment of the copolymers, since it is a non-toxic polymer approved by FDA for use in various pharmaceutical formulations[52-54]. Its unique physico-chemical properties such as high-water solubility, high flexibility, and large exclusion volume provide good stealth properties to the nanoparticles, thus increasing their circulation time. Poly(N-vinyl-2-pyrrolidine) (PVP) and poly (acrylic acid) are other polymers used as the shell forming agents[55,56].

The hydrophobic segment forming polymers include poly(propylene glycol) (PPO, Pluronics)[57], poly(aspartic acid)[58], poly(β-benzyl-L-aspartate) (PBLA)[59], and poly(ester)s such as poly(lactic acid) (PLA) [60,61], poly(ε-caprolactone) (PCL)[62,63]and poly(trimethylene carbonate) (PTMC)[64].

The CMC of the amphiphilic block copolymeric micelles is found to be in the order of $10^{6}$-$10^{7}$ M, while that of low molecular weight surfactant is on the order of $10^{3}$-$10^{4}$ M[65]. Thus, the surfactant micelles are more prone to dissociation at lower concentrations while polymeric micelles are more stable. As the polymeric micelles are subjected to dilution upon IV administration, this lower CMC becomes advantageous in maintaining the micellar structure facilitating prolonged circulation the blood stream.

### 4.2. Techniques for the preparation of PNPs

PNPs can be prepared by many techniques, but the choice of the method depends on a number of factors such as the desired particle size, particle size distribution, area of application etc.

PNPs can be conventionally prepared either from preformed polymers or by direct polymerization of monomers using classical polymerization or polyreactions. Methods for the preparation of PNPs from preformed polymers include solvent evaporation, salting out, dialysis, and supercritical technology. On the other hand, PNPs can be directly synthesized by the polymerization of monomers using various polymerization techniques such as micro-emulsion, mini-emulsion, surfactant-free emulsion, and interfacial polymerization[47].

## 5. Stimuli Responsive Polymers:

Stimuli responsive polymers are defined as "polymers that undergo relatively large and abrupt, physical or chemical changes in response to small external changes in environmental conditions". They recognize a stimulus as a signal, judge the magnitude of this signal, and then change their chain conformation in direct response[66].

There are many stimuli that modulate the response of polymer systems which include:

**A. Physical stimuli:**

a) Temperature

b) Electric field

c) Magnetic field

d) Ultrasound

e) Magnetic stress

**B. Chemical stimuli:**

a) pH

b) Enzymes

c) Ionic strength

d) Redox potential

Some systems have been developed to combine two or more stimuli responsive mechanisms into one polymer systems, so-called "the dual responsive polymer systems". For example, temperature sensitive polymers may also respond to pH changes by modification of the polymeric structure.

### 5.1. Temperature Sensitive Polymers:

Temperature is the most widely used stimulus in environment responsive polymer systems. The change of temperature is not only easy to control, but also easily applicable both *in vitro* and *in vivo*.

One of the unique properties of temperature-responsive polymers is the presence of a "critical solution temperature (CST)". CST is the temperature at which the phase of polymer is discontinuously changed according to their composition. The polymer may either have "Lower Critical Solution Temperature (LCST)" or "Higher Critical Solution Temperature (HCST)".

At lower temperatures, hydrogen bonds between the hydrophilic segments of the polymer chain and water molecules are dominant making the polymer more soluble in water. With the increase of temperature, partial displacement of water from the polymer coil occurs, weakening the hydrogen bonds and increasing the hydrophobic interactions between the hydrophobic segments of the polymer macromolecules[67-69]. Consequently, the intra- and intermolecular hydrogen bonding between the hydrophobic parts of the polymer molecules are favoured, resulting in polymers collapse, aggregation and phase separation. The LCST phenomenon is reversible, upon cooling the thermosensitive polymers become soluble again (Fig 3).

Other characteristic of polymers making them to respond to temperature changes is the presence of hydrophobic and hydrophilic groups in their structure, due to which they show temperature responsive micellization and gelation[70]. They have their sol-gel transition under body temperature and gel-sol phase transition around 50°C in relatively high concentration range. The mechanism of sol-gel transition was elucidated as an increased micelle volume change, which causes crystal like packing of micelles. As the temperature increases over the critical threshold, the micellar structure changes from spherical to cylindrical with the subsequent release of entrapped materials. The biopolymers and polypeptides respond to temperature by change in their three-dimensional structure with the change in temperature and thus release the drug entrapped[71,72].

By virtue of their ability to respond to the critical temperature, the polymers release the entrapped drug at the target site around this temperature which can be exploited in delivering drug to tumors, by adjusting critical temperature to required range. Based on the mechanism by which they respond to temperature, the thermo responsive polymers are classified into (Table 2):

1. Polymers based on LCST
2. Polymers based on amphiphilic balance
3. Biopolymers and artificial polypeptides

### Modifying temperature sensitive polymers to respond to required temperature:

Rapid responsive kinetics could be controlled by molecular level design.

1. Attaching hydrophobic moieties to the polymer could increase the deswelling rate, reducing the hydrophobic aggregation on the surface which suppresses layer formation.
2. De-swelling can also be achieved by constructing the molecular architecture as a comb rather than a linear structure.
3. The LCST of a temperature sensitive polymer (PNIAAm) can be increased by copolymerization with a small ratio of hydrophilic monomers like acrylamide.
4. A small ratio of hydrophobic constituent (N-butylacrylamide) can decrease the LCST of NIPAAm and hence can increase its temperature sensitivity.
5. The hydrolytically sensitive lactate ester side groups can be introduced to control the LCST of temperature sensitive NIPAAm based random copolymer by hydrolysis of lactate ester side groups.
6. LCST is dependent on molecular weight and concentration. For example, increasing the polymer length or polymer concentration decreases the LCST of PNIPAM and PVCL.
7. Salts are known to lower the LCST of PNIPAM and PVCL. As the concentration of salts in the solution increases, the LCST decreases, as more competing ions for H-bonding and hydrophobic interactions are available.
8. Small amount of alcohols also decreases the LCST.
9. Proteins, such as insulin and bovine serum albumin, have been found to increase the LCST, because of the increased hydrophilicity of the polymer-protein complex.
10. Surfactants, depending on their hydrophobic chain length and concentration, either decrease or increase the LCST.

### 5.2. pH Sensitive Polymers:

The pH responsive polymers consist of pendants that can accept and donate protons in response to the environmental changes in pH. As the environmental pH changes, the degree of ionization of the polymer bearing weakly ionisable groups is dramatically altered at a specific pH that is called as pKa. The rapid change in the net charge of the pendant groups causes an alteration of the hydrodynamic volume of the polymer chains. The osmotic pressure exerted by mobile counterions neutralizing the network charges results in a transition from a collapsed state to expanded state[73].

Polymers with ionisable groups in their backbone form polyelectrolytes in the aqueous system. The pH-sensitive polyelectrolytes are of two types: weak polyacids and weak polybases (Table 3). The other classes of pH sensitive polymers include pH responsive bio-degradable polymers and artificial polypeptides. The ionisable pendant group of weak polyacids can be either a carboxylic group or a sulphonamide group. Weak polyacids, for example polyacrylic acid, accept protons at low pH and release protons at neutral or higher pH[12]. On the other hand, polybases such as poly (N,N'-dimethylaminoethyl methacrylate) get protonated at high pH and positively ionized at neutral or low pH[13]. Hence, the selection between polyacids and polybases should be tailored according to the desired application.

### Adjusting for Critical pH:

Adjusting the appropriate critical pH, at which reversible conformational changes of polymer chains occur, is an important factor for the pH responsive polymer-based applications (Fig 4). The pH over which a reversible phase transition occurs, can be generally modulated by two strategies:

(i) Selecting the ionisable moiety with a pKa matching the desired pH range.

(ii) Changing critical pH by incorporating a hydrophobic moiety into the polymer backbone

The intrinsic pKa values of an ionisable moiety should be given first consideration in the selection of a proper pH-responsive polymer for the desired application. Poly(L-lysine) undergoes a pH responsive-phase transition, but its pKa is too high (~10.5) to use in biomedical application requiring its transition near physiological pH. On the other hand, poly(histidine) can be an appropriate candidate as a pH-responsive polymer, because it has its pKa at 6.0[74]. The pKa is related to critical pH at which half of the ionisable groups are ionized. However, the conformational changes which occur at critical pH do not occur at the pKa. This transition is governed by the balance between electrostatic repulsion and hydrophobic interactions.

Incorporating a hydrophobic moiety might increase the critical pH, because the stronger hydrophobic interactions are present and so a higher electrostatic repulsion resulting from more ionisable group is required.

### 5.3. Double targeting:

Double targeting is a universal approach for targeting drug sensitive, resistant tumors. One of the strategies, taking in to account the issue related to both MDR and tumor tissue heterogeneity, is to use tumor cell non-specific interactions that can be activated by tumor microclimate such as extracellular pH (pHe) along with triggered release in the endosomes (pHendo). Here, both the ligand mediated, and physical targeting strategies of active targeting are combined in targeting the drug more specifically at tumor site. A review by Lee et al., has an in-depth discussion on double targeting as a universal approach[75].

pH sensitive polymeric micelles in which the ligand is repositioned on the micelle surface as a response to the changes in pH were developed. One example includes the mixed micelle prepared with polyHis-b-PEG and PLLA-b-PEG(MW=1000)-b-polyHis-biotin, which is multifunctional. The hydrophobic micelle core is formed by PLLA, poly(L-lactic acid), polyHis and the hydrophilic PEG shell. At the interface of the hydrophobic core and hydrophilic PEG shell: a shorter polyHis block, neighbouring hydrophilic PEG and biotin, is present on the hydrophilic side. The interfacial short polyHis block causes PEG chain bending and the biotin burying in the PEG shell (Fig 5).

At and above pH 7.2, the micelle is stable and hides the conjugated biotins. As the pH falls below 7.2, an increase in ionization of polyHis can be observed, beginning with ionization of the interfacial polyHis; leading weaking of its interaction with the hydrophobic core and the expansion of the PEG-b-polyHis-biotin.

pH 7.0 is considered as the critical point for expansion as demonstrated by a pH-dependent turbidity of the micelle solution containing a tetrameric protein, avidin, with four binding sites for biotin. As the pH of the solution falls from 6.8

to 6.0, the relative transparency of the solution is reduced to 10%. This is caused by the ionization of polyHis block, resulting in destabilization and escape of polyHis from the micelle.

At a slightly lower environmental pH (pH~7.0, pHe), biotin is exposed on the surface of micelle and can interact with cells, facilitating biotin receptor mediated endocytosis. As the pH is furthered lowered below 6.5, the micelle is destabilized, and the endosomal membrane is damaged enhancing the drug release in the cytosole. The pH responsive micelle presents an opportunity to selectively enhance the cytotoxicity at the acidic pH of the tumor[75].

### 5.4. Block copolymers with dual-response to both temperature and pH:

For better targeting efficiency and treatment efficacy, extensive efforts for the development of dual responsive drug carriers have been made, such as PNPs responsive to both changes in temperature and pH. Thermo-responsive block copolymers show not only special selectivity in the body due to EPR effect but also increased retention at a target site with local heating. On the other hand, pH sensitive block copolymers can self-assemble to form micelles which exhibit high drug loading capacities and release the drug in a pH dependent fashion. Combining both the strategies provides better targeting.

Cholesterol grafted poly (NIPAAm-co-DMAAm-co-UA) was synthesized and utilized to encapsulate a highly hydrophilic drug (paclitaxel), and the micelle based on the copolymer exhibited a useful pH-induced thermo selectivity. Wei et al. developed thermo and pH dual-responsive micelles of poly (UA-b-NIPAAm) as a drug delivery system for Prednisone acetate, which showed a dramatic thermo-responsive switching behaviour and a unique pH-responsive behaviour [80]. Jiang's group reported a kind of nanoparticles assembled from P(NIPAAm-co-AA)-b-PCL, which demonstrated to be responsive to both temperature and pH in a suitable window for targeted anti-cancer drug delivery[76].

Combining a temperature responsive polymer such as PNIPAAm with a pH sensitive polymer such as PAAc can offer another mechanism for pH responsive characteristics. The LCST of PNIPAAm can be controlled by incorporating hydrophilic moieties into the polymer backbone. The pH dependent ionisable groups in pH responsive moieties switch hydrophilicity/hydrophobicity, leading to the LCST change. For example, the LCST above body temperature can drop to below body temperature by ionization of even small fraction of pH responsive moieties. Although the phase transition comes from the LCST of PNIPAAm, the controlling stimulus is the pH change in the system.

## 6. Drug Loading Into PNPs:

The three general methods of drug loading into polymeric micelles include: (i) chemical conjugation, (ii) physical entrapment or solubilisation, and (iii) polyionic complexation[77].

**(i) Chemical conjugation**: Drug incorporation into polymeric micelles via chemical conjugation was first proposed by Ringdorf's group in 1984. According to this approach, a drug is chemically conjugated to the core-forming block of the copolymer via a carefully designed pH or thermosensitive linker that can be cleaved to release a drug in its active form. The polymer-drug conjugate then acts as a prodrug which self assembles into a core-shell structure.

The rate of drug release, and therefore, the effectiveness of the prodrug is controlled by the nature of the polymer-drug linkage and the stability of the drug-conjugate linkage. For instance, recent work by Kataoka's group proposed pH-sensitive polymer micelles of PEO-b-poly (aspartate hydrazone doxorubicin), in which doxorubicin was conjugated to the hydrophobic segments through acid-sensitive hydrazone linkers that are stable at extracellular pH of 7.4 but degrade and release free drug at a lower pH.

**(ii) Physical entrapment**: For hydrophobic drug molecules, the physical incorporation or solubilization of drugs within block copolymer is preferred over micelle-forming polymer drug-conjugates. Indeed, many polymers and drug molecules do not contain reactive functional groups for chemical conjugation and therefore, specific block copolymers have to be designed for a given type of drug. In contrast, a variety of drugs can be physically incorporated into the core of the micelles, by engineering the structure of the core forming segment.

Different loading methods for physical entrapment include dialysis, oil in water emulsification, direct dissolution, or solvent evaporation techniques. Depending on the method, drug solubilisation may occur during or after micelle assembly. The loading capacity of polymeric micelles is influenced by several factors, including both the structure of core-forming block and a drug, molecular characteristics of the copolymer such as composition, molecular weight, and the solution temperature.

**(iii) Poly-ionic complexation**: Charged therapeutic agents can be incorporated into block copolymer micelles, through electrostatic interactions with an oppositely charged ionic segment of block copolymer. The approach was independently put forward by Kabanov and Kataoka in 1995 and now is widely used for the incorporation of various polynucleic acids into block ionic complexes, for the development of non-viral gene delivery systems.

Ionic block lengths, charge density, and ionic strength of the solution affect the formation of stable block ionomer complexes. The pH and salt sensitivity of such block ionomer micelles provide a unique opportunity to control the triggered release of the drug. Furthermore, block ionomer complexes are believed to account for the polyion interchange reactions which are believed to account for the release of the therapeutic agent.

## 7. Characterization of the PNPs:

The therapeutic efficacy of the nanoparticle is determined by its physicochemical properties which include size, size distribution, surface and bulk morphology, surface chemistry, surface charge, drug encapsulation efficiency, stimuli responsiveness, and physical and chemical status of the encapsulated drug[77]. Various analytical methods are employed in characterizing PNPs which are summarized in Table 4.

### pH/ temperature induced phase transitions:

**Transmittance measurements:** The phase transition at various temperatures and pH can be traced by monitoring the transmittance of a 500nm light on a Spintronic 20 spectrophotometer (Baush & Lomb). 5% w/w aqueous solution of the polymer are taken, and the temperature is gradually raised from 15 to 70°C in increments of 2°C every 10min. To observe their pH/temperature dependence, the phase transitions of polymers in phosphate buffer solution versus temperature at two pH values (<7 and 7.4) are measured[78]. Other techniques include FT-IR and $^{13}$C-NMR measurements.

### Encapsulation and loading efficiency:

The drug loaded nanoparticles are separated from aqueous suspension by ultracentrifugation and the amount of free drug

in the supernatant can be determined by reverse phase HPLC. Entrapment efficiency and loading capacities can be calculated using the formulas.

$$Encapsulation\ effeciency\ (\%) = \frac{Actual\ amount\ of\ drug\ loaded\ in\ nanoparticles}{Theoritical\ amount\ of\ drug\ loaded\ in\ nanoparticles} \text{ X } 100$$

$$Loading\ capacity\ (\%) = \frac{Actual\ amount\ of\ drug\ loaded\ in\ nanoparticles}{Total\ weight\ of\ nanoparticles} \text{ X } 100$$

### *In vitro* drug release:

The nanoparticles are suspended in phosphate buffer solution at various pH at the same temperature for pH sensitive PNPs and the aliquots are withdrawn at regular intervals and analysed by HPLC. In case of thermosensitive PNPs, the pH is kept constant, and temperature is varied, and the samples are analysed for the % of drug release.

## 8. Pharmacokinetics and biodistribution:

Chemotherapeutic agents, when administered into the body, extravasate to various tissues, affecting them almost indiscriminately causing serious side effects and are rapidly removed from the body. Furthermore, many drugs have low stability and are degraded in the body forming toxic metabolites. An example is doxorubicinol, a major metabolite of doxorubicin which causes cardiac toxicity. These impediments to the therapeutic use of these agents can be mitigated by entrapping them in PNPs[79]. Incorporation of these agents into PNPs drastically alters biodistribution and pharmacokinetics in the body, which is crucial for the drug action. The pharmacokinetics and biodistribution of the entrapped drug are governed by the surface properties, size, and stability of the nanoparticle and less effected by the properties of the drug.

From the point of size, the nanoparticles are small enough to be systemically administered and large enough to escape from renal excretion. On the other hand, they are not large enough to be phagocytised by RES system. Stealth properties can be induced by attaching PEG chains, thus increasing their circulation half-life in the body. Because of the EPR effect of the tumor endothelial cells, these particles get extravasated into the tumor interstitium. Furthermore, responsiveness to stimuli enhances the drug targeting ability by releasing the drug when encountered with a particular pH or temperature. Thus, drug is released within a limited region of tumor, reducing the toxic effects of the cytotoxic agent on the healthy cells.

## 9. Conclusion:

The impact of nanotechnology for cancer therapy is discernible. The advantages of nanocarriers over current treatment regimens for cancer therapy include lower toxicity due to entrapment of cytotoxic agent, and improved bioavailability due to altered pharmacokinetics. Surface engineering, such as PEGylation, further enhances their circulation half-life, thus decreasing the nanoparticle clearance by phagocytic cells of the body. Recent progress in polymer science had led to the development of PNPs. Chemical modification of the polymeric chains brings about stimuli responsiveness, which fosters tissue specific drug delivery of anti-cancer agent to tumors. The LCST or critical pH should be controlled to match the desired environmentally critical condition, after selecting the appropriate polymers. Rapid response to stimuli is another important factor to be considered in designing their molecular structure. Biocompatibility should also be given due consideration. Stimuli responsive nanoparticles with dual and double response have a synergistic effect and are believed to more efficient in targeting. Even though the stimuli responsive polymers are attractive for their potential, they have to overcome several barriers such as rapid response, mechanical strength, reproducibility, non-toxicity, and so on. Research trials are in progress to overcome these barriers and develop stimuli responsive polymers with minimal side effects.

**Table 1:** MDR transporters and their chemotherapeutic substrates

| MDR transporter | Chemotherapy substrates |
|---|---|
| MDR1 | Vinca alkaloids, Anthracyclines, Taxanes, Actinomycin-D, Epipodophyllotoxins, Steroids |
| MRP1 | Doxorubicin, Daunorubicin, Vincristine, Etoposide, Methotrexate |
| MRP2 | Vinca alkaloids, Cisplatin, Methotrexate |
| MRP3 | Etoposide, Teniposide, Methotrexate, Vincristine |
| MRP4 | Methotrexate, Purine antimetabolites |
| MRP5 | Purine antimetabolites |
| BCRP | Mitoxantrone, Camptothecins, Anthracyclines |

**Table 2:** Classification of polymers based on their mechanism of response to temperature change

| Class | Mechanism | Examples | Temp (°C) |
|---|---|---|---|
| Polymers based on LCST | Phase transition from the soluble to the insoluble state | Poly(N-isopropylacrylamide) [PNIAAm] | 32 |
| | | Poly(N, N'-diethylacrylamide) [PDEAAm] | 25-35 |
| | | Poly(2-carboxyisopropylacrylamide) [PCIAAm] | 32 |
| | | Poly(N-(L)-1-hydoxymethylpropyl methacrylamide) [P(HPMAAm)] | - |
| | | *Poly(N-acryloyl-N-alkylpiperazine) | |
| | | *Copolymers of N-acryolyl-N'-alkylpiperazine (methyl and ethyl) with | 37 |

| | | | |
|---|---|---|---|
| | | methacrylamide (PMAAm)<br>Poly(N-vinylisobutylamide)<br>Poly(vinylmethylether)<br>Poly(N-vinylcaprolactam)<br>Poly(dimethylaminomethylmethacrylate) | -<br><br>-<br>-<br>-<br>- |
| Polymers based on amphiphilic balance | Sol↔gel transition and Temp responsive micellar structural transitions | *PEO-*PPO-PEO triblock copolymer<br>Sol ↔ gel transition<br>Gel ↔sol transition<br>Poly (2-ethoxyethyl vinyl ether-b-2-hydroxyethylvinyl ether) [P(EOVE-b-HOVE)] | <br>37<br>~50<br>- |
| Biopolymers and artificial polypeptides | Swelling ↔ de-swelling due to intermolecular interactions and conformational changes | Gelatin, agarose, gellan derivatives, chitosan derivatives, recombinant artificial elastin like polypeptides | adjustable |

*PEO = Polyethylene oxide, *PPO = Polypropylene oxide

**Table 3;** Classification of pH sensitive polymers based on the mechanism of drug release

| Class | Mechanism | Examples | pKa |
|---|---|---|---|
| Polyacids | Transform into polyelectrolytes at higher pH with electrostatic repulsions between molecular chains which give a momentum along with hydrophobic interactions to govern precipitation/ solubilisation or swelling/deswelling of molecular chains. | **Polyacids bearing carboxylic groups**: Poly(acrylic acid) (PAAc)<br>Poly(methracrylic acid) (PMAAc)<br>Poly(2-ethyl acrylic acid) (PEAAc)<br>Poly(2-propyl acrylic acid) (PPAAc)<br>**Polyacids bearing sulphonamide group** | 4-5<br><br><br><br>3-11 |
| Polybases | Abrupt precipitation above a specific pH due to deprotonation of functional groups followed by hydrophobic molecular interactions. | **Polybases with amine groups:**<br>Poly (N,N'-dimethyl aminoethyl methacrylate) (PDMAEMA)<br>Poly (N,N'-diethyl aminoethyl methacrylate) (PDEAEMA)<br>**Polybases with pyridine group:**<br>Poly (4 or 2-vinylpyridine)<br>**Polybases with imidazo group:** poly (vinyl imidazole)<br>**Polybases with piperazine group:**<br>*Poly(N-acryloyl-N'-alkylpiperazine) | |
| pH responsive degradable polymers | Structural degradation at certain pH. | Poly (ortho ester)<br>Poly (β-amino ester) | < 6.5 |
| Biopolymers and artificial polypeptides | pH responsive phase transition. | - Alginate<br>- Chitosan<br>- Synthetic polypeptides consisting of amino acids with ionisable pendant groups such as Cysteine<br>- Aspartic acid<br>- Glutamic acid<br>- Histidine<br>- Lysine<br>- Arginine | <br><br>8.4<br><br>3.9<br>4.1<br>6.0<br>10.5<br>12.5 |

**Table 4:** Various parameters for the characterization of polymeric nanoparticles

| Property | Analytical method |
|---|---|
| Presence | Dark field optical microscopy. |
| Size | DLS, Static light scattering, Ultrasonic spectroscopy, Turbidimetry, NMR, Single particle optical sensing, FFF Hydrodynamic fractionation, Filtration. |
| Morphology | TEM, SEM, AFM |
| Surface charge | Electrophoretic light scattering, U-tube electrophoresis, Electrostatic-FFF |
| Surface hydrophobicity | Hydrophobic interaction chromatography |
| Surface adsorbates | Electrophoresis |
| Density | Isopycnic centrifugation, Sedimentation-FFF |
| Interior structure | Freeze fracture SEM, DSC, X-ray diffraction, NMR |

DLS; Dynamic light scattering, NMR; Nuclear magnetic resonance, FFF; Field fractionation, TEM; Transmission electron microscopy, SEM; Scanning electron microscopy, AFM; Atomic force microscopy, DSC; Differential scanning calorimetry.

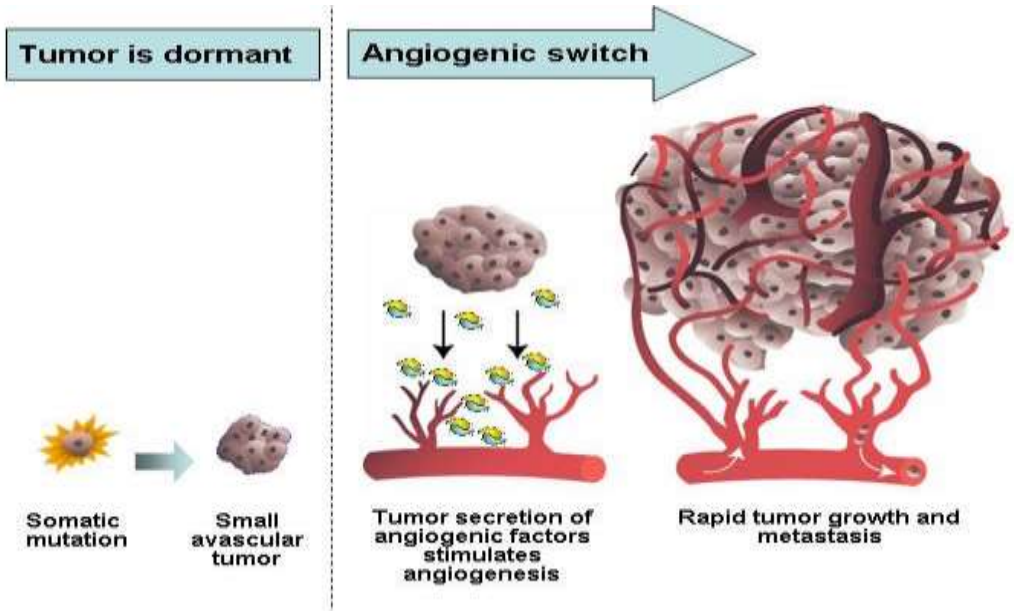



**Figure 1;** Schematic representation of the formation of new blood vessels (angiogenic switch).

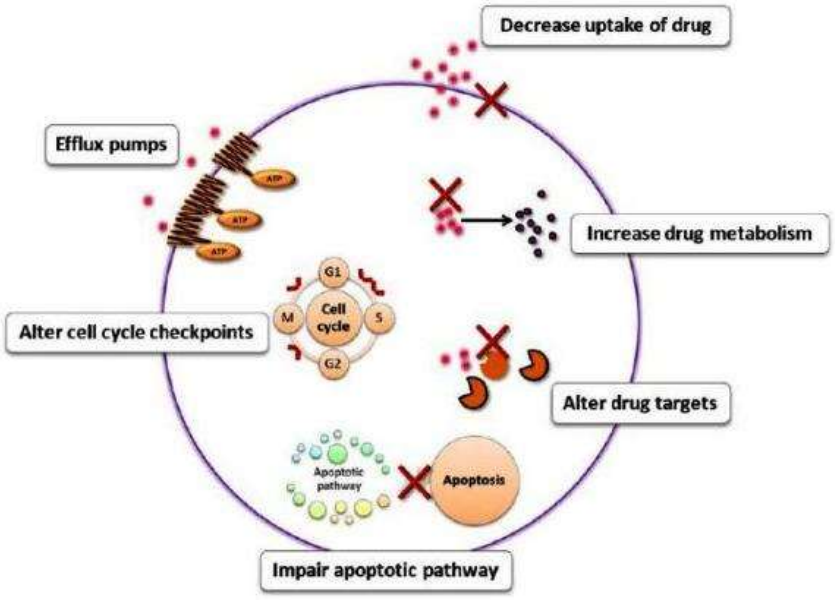



**Figure 2: Various mechanisms of drug resistance exhibited by the tumor tissue.**

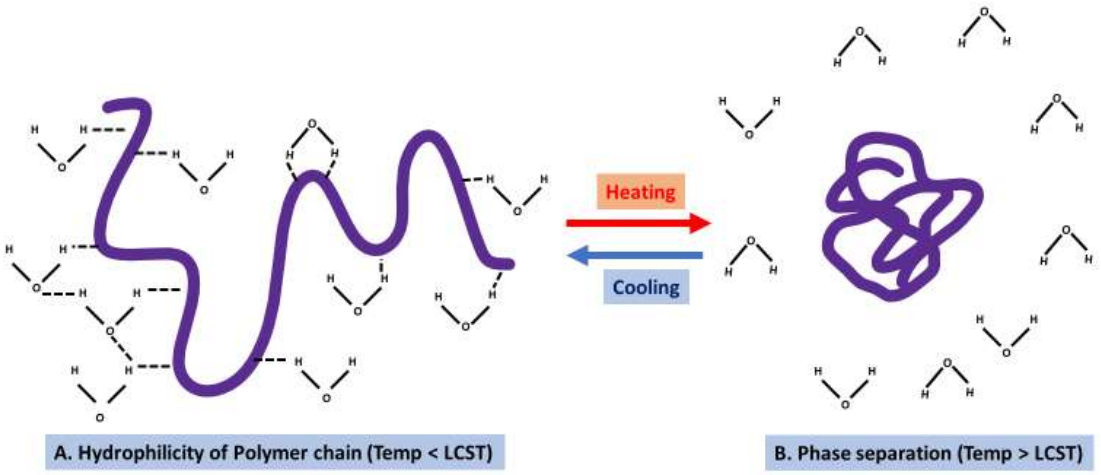



**Figure 3: Temperature sensitivity and structural changes of the thermosensitive polymer chain.**

A. The hydrophilic segments of the polymer chain and the water molecules are bonded at low temperatures.

B. Upon heating, the water molecules are displaced and hydrophobic interactions within the polymer leads to phase separation.

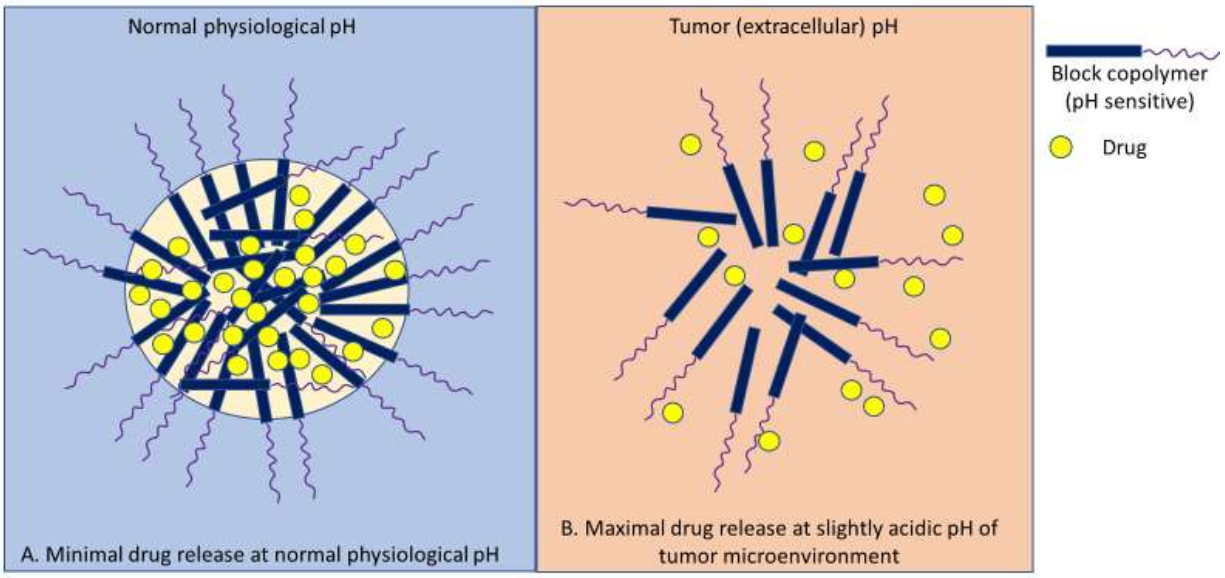



**Figure 4: Schematic of drug release from pH sensitive block copolymers**

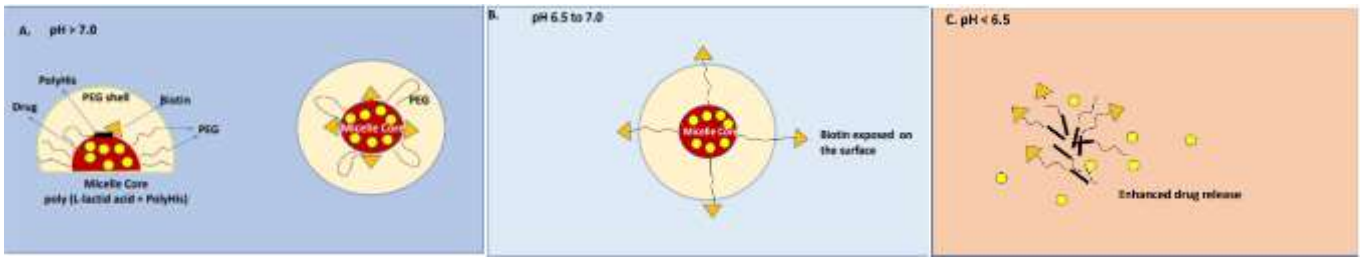



**Figure 5: Schematic of the pH sensitive biotin repositioning on the micelle.**

(A). Conjugated Biotin anchored on the micelle core at pH>7.0.

(B). At pH between 6.5 and 7.0, ionization of polyHis occurs leading to its reduced hydrophobic interaction with the micelle core. The PEG-b-polyHis-biotin expands, and biotin is exposed. Interaction of biotin with biotin receptors on the cell surface facilitates endocytosis.

(C). The micelle destabilizes at pH < 6.5 facilitating an enhanced drug release.

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