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Hydrogel-nanoparticles as versatile system for controlled drug delivery

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Sommario

La lesione del midollo spinale rimane, ad oggi, una delle più problematiche patologie neurologiche a causa dei conseguenti effetti a lungo termine. È stimato, che circa 330.000 cittadini europei siano affetti da tale patologia, che, anche in Italia, rimane una delle maggiori cause di disabilità.

Tipicamente queste lesioni derivano da eventi traumatici, come incidenti d'auto o in ambito sportivo, come anche come conseguenza di eventi violenti, come ferite da arma da fuoco. In aggiunta a queste cause, poi, occorre considerare anche fonti non traumatiche, come la presenza di patologie quali la poliomielite, spina bifida o ancora tumori.

Fino ad ora non esiste un trattamento risolutivo per le lesioni del midollo spinale: anche i trattamenti medici che vengono somministrati immediatamente dopo la lesione, inclusa l'immobilizzazione e il rinforzo per la stabilizzazione della colonna, possono solo aiutare a minimizzare il danno alle cellule nervose. Tale patologia, infatti, modifica drasticamente l'ambiente del midollo spinale, in quanto, durante le prime settimane post-infortunio, le cellule del sistema immunitario vengono richiamate nel sito del danno, vengono rilasciate sostanze tossiche e si forma una cicatrice che genera una discontinuità permanente nella trasmissione di informazioni da e per il sistema nervoso centrale.

In questo contesto, l'ingegneria tissutale tende ad essere ampiamente configurata come tecnologia promettente per la medicina rigenerativa e per la cura della salute. L' "ingegneria dei tessuti" fonda i suoi principi sullo studio e l'applicazione di una intelligente combinazione di cellule e materiali in grado di poter sostituire parti mancanti o riparare quelle danneggiate di tessuti viventi. In generale, si tratta di "costruire" una struttura biologicamente attiva e compatibile, capace di trasportare farmaci e/o cellule appropriati che, ad esempio nel caso della lesione spinale, possano portare alla soppressione dell'infiammazione (prevenendo così la diffusione della lesione), alla protezione delle

cellule nel sito di lesione da ulteriori danni attraverso il rilascio di sostanze terapeutiche e alla sostituzione di cellule nervose morte con nuove, capaci di stimolare la riconnessione tra le fibre nervose interrotte.

Le nuove strategie nella medicina rigenerativa confermano un grande interesse nei confronti degli idrogeli come efficienti veicoli di trasporto sia di cellule che di principi attivi: strutture polimeriche tridimensionali dotate di elevata biocompatibilità e possibilità di rilascio controllato dei farmaci e delle cellule caricate.

Focalizzando l'attenzione sull'aspetto del drug delivery, gli idrogeli non si rivelano in realtà la migliore opzione per il rilascio di farmaci, sia perché possono essere caricati solo di farmaci idrofilici, essendo strutture a base acquosa, sia perché spesso il rilascio di farmaci per via diffusiva è spesso troppo veloce per ottenere l'attesa efficacia terapeutica. D'altra parte, le nanoparticelle si sono dimostrate essere degli efficienti veicoli farmacologici, ma per via delle loro dimensioni, tendono a spostarsi dal luogo della lesione e diffondersi in tutto l'organismo, perdendo così parte della loro efficacia.

Per questo motivo, soprattutto negli ultimi decenni, la ricerca ha suggerito la combinazione di questi due sistemi, nanoparticelle e idrogeli, per superare le rispettive limitazioni e ottenere un sistema in grado di ottenere il miglior effetto terapeutico possibile *in situ*, assicurando il corretto rilascio di farmaci di qualsiasi genere (idrofilici e idrofobici) nel tempo, evitando sotto o sovradosaggi e i conseguenti effetti collaterali.

In questa tesi si è studiata la possibilità di sintetizzare nanoparticelle polimeriche, biodegradabili e biocompatibili, basate su polimero PEG-b-PLA, secondo diverse metodologie di produzione. Tali particelle si sono dimostrate anche adatte per essere funzionalizzate con una carica netta superficiale, conferita da surfattanti ionici fisicamente adsorbiti sulla superficie della particella. Questa caratteristica ha permesso la creazione, da un lato, di nanocluster, dall'altro l'interazione elettrostatica con idrogeli carichi, come AC1, AC6 e AC&+CMC.

Il risultato è stata la realizzazione di un sistema composito per il drug delivery che permettesse sia la carica di farmaci idrofilici e/o idrofobici che la possibilità di modulare le velocità di rilascio di tali composti grazie alle interazioni elettrostatiche tra nanoparticelle e idrogeli.

Abstract

Spinal Cord Injury (SCI) remains one of the most devastating condition among neurological diseases, due to its pathologic consequences. It is estimated that this lesion affects about 330.000 European people, and it is one of the leading causes of disability in Italy.

Usually, these injuries are the result of traumatic events (for example, a motor vehicle accident or a sport injury or by violence such as gunshot wound), but many also are the outcome of non-traumatic causes as consequence of medical treatments or diseases, such as polio and split spine or the presence of tumoral mass.

Currently, there is no effective strategy for the treatment of SCI: medical care immediately after the lesion, including immobilizing and bracing to stabilize the spine, can best help to minimize the damage of neural cells. SCI involves also different kind of damage to distinct types of cells; the environment if spinal cord changes drastically during the first few weeks after injury, because immune cells flow in, toxic substances are released and a scar is formed, which generates permanent interruption of information transmission to and from the central nervous system.

About this, tissue engineering is a widely accepted as being the future in regenerative medicine and health care. It studies the smart combination of cells and materials to replace damaged or missing parts of living tissues: it points toward the synthesis of a biological active and compatible structure able to carry functional drugs and cells that, for example in SCI, can suppress the damaging inflammation (preventing spread of injury), protect the cells at the injury site from further damage by releasing therapeutic substances and replacing dead nerve cells with new one, capable of promoting reconnection between interrupted nerve fibers.

Emerging strategies in regenerative medicine confirm a very strong interest in hydrogel as great candidates for both cell and drug delivery, allowing the building of biocompatible three-dimensional polymer network with cells and drug directly included inside gel and then released in a controlled way.

Focusing on the drug delivery aspect of those systems, hydrogels are not the best option as drug delivery systems because they can load just hydrophilic compounds, due to their nature, and release is often too fast to achieve the correct therapeutic profile. On the other side, polymeric nanoparticles have demonstrated to be very effective carrier for drug delivery, but mainly because of their dimensions, tend to diffuse all over the organism, losing part of their efficacy.

For this reason, last decades study, suggest that the combination of those two biomedical devices, hydrogels and nanoparticles, to achieve the best therapeutic effect *in situ*, assuring the correct delivery of drugs of any nature (hydrophilic or hydrophobic) in time, avoiding under and overdosing and subsequent side effect.

In this thesis, it is studied the possibility to synthetize biodegradable and biocompatible polymeric nanoparticles, based on PEG-b-PLA copolymer, via different production methods. Those nanoparticles have also demonstrated to be suitable for functionalization with well-defined surface charge, through physical absorption of ionic surfactants on particle surface. This feature allows to create nanocluster, on one side; in the other side to relate with charged hydrogel network, such as AC1, AC6 and AC6+CMC through electrostatic interaction, creating a drug delivery system with the modulable release capability of multiple drugs loaded.

1 Nanomedicine

The European Science Foundation (ESF) defines nanomedicine as the science and technology of diagnosis, treating and preventing disease and traumatic injury, of relieving pain and preserving and improving human health, using molecular tool and molecular knowledge of human body (ESF 2004).

The early genesis of concept of nanomedicine comes from the revolutionary idea of nanotechnology, introduced for the first time by the Nobel physicist Richard P. Feynmann in 1959. The idea was to create tiny nanorobots and machines that could be designed, manufactured and introduced into the human body to perform cellular repairs at the molecular level (Freitas 2005). Indeed, the term nanotechnology refers to the ability to measure, design and manipulate material at atomic, molecular or supramolecular level, in order to understand, create and apply the resulting nanodevices able to perform a specific function in range of 1-100 nm. This is the typical size range that involves nanotechnology, although often it can be expanded to include materials that are below 1 μm.

Nanotechnology has been embraced by multiple industrial sectors for application in field of electronic storage systems, biotechnology, magnetic separation, targeted drug delivery and gene delivery vehicles (Boulaiz et al. 2011). Development in nanotechnology lead to the discovery of new nanomaterials, whose physiochemical properties are strictly connected to their surface-to-volume ratio and differs largely from their bigger counterparts. Those novel properties make them excellent candidates for biomedical applications, given the range of biological processes that occurs at nanometer scale. In addition to that we can also include biocompatibility and biodegradability and quite simple adaptation to various clinical situation and purposes.

Therefore, it's evident that the application of nanotechnology to screening, diagnosis and treatment of diseases, which is generally referred to as nanomedicine. In short, it can be considered as the refinement or molecular medicine, integrating innovations in genomics and proteomics on the road of more personalized medicine. (Boulaiz et al. 2011).

The impact of nanotechnology in medical field can be mainly seen in diagnostic methods, drug release techniques and regenerative medicine and all those applications, that are nowadays pursued, are very close to fruition, with an almost inevitable success. Indeed, beside the importance of a prompt diagnosis that is well known, the conventional drugs suffer from the major limitation of adverse effect, the result non-specificity of their action and from a leak of effectiveness due to improper dosages. Nanomedicine offers the possibility to design novel drugs with greater cell specificity and new drug-release methods, which act selectively on specific targets and protect the drug from degradation *en route*. This allows the administration of smaller but more effective doses, minimizing adverse effects. The optimization of drug formulation is also possible, obtaining a better drug solubility and altering pharmacokinetics to sustain the release and prolonging its bioavailability.

Nanomedicine applications are related to three different principal areas: analytical and diagnostic tools, regenerative medicine and finally drug delivery.

1.1 Drug Delivery

A drug delivery system (DDS) is defined as a formulation or device which allows the introduction of a therapeutic compound inside the human or animal body, in order to improve its efficacy and safety by controlling the rate, time and place of drug release. It can be seen as a sort of intermediate system between the patient and the drug, including both administration and release of active ingredient and the subsequent transport across biological membranes to the site of action (Davis 2000).

As discussed by Rossi F. *et al.,* 2016, the method by which a drug is delivered can influence significantly its efficacy. Indeed, some drugs have an optimal concentration range above or below which can be toxic or producing no therapeutic effect at all. Some traditional drug delivery methods, such as pills or intravenous injection, cause peaks of drug concentration that are outside the therapeutic one and consist in over dosing of active compound, that although are necessary to achieve a therapeutic level. On the other side, while the concentration profile diminishes over time, it can go rapidly under the desired range, being no more effective.

Thus, the aim of drug delivery systems is to avoid under and over dosing, maintaining the drug level inside therapeutic desired level, optimizing drug administration. In this way, it's possible to deliver a certain amount of active substance for a prolonged period of time with a great selectivity towards the target area inside the body.

The therapeutic window, or therapeutic range, is the set of concentration values at which the drug has been shown to be effective without causing toxic effects. This range is delimited at the top by the Minimum Toxic Concentration (MTC) of active compound and at the bottom by the Minimum Effective Concentration (MEC).

If drug level exceeds MTC, it will surely express a toxic effect, while if it's lower the result is a complete therapeutic failure.

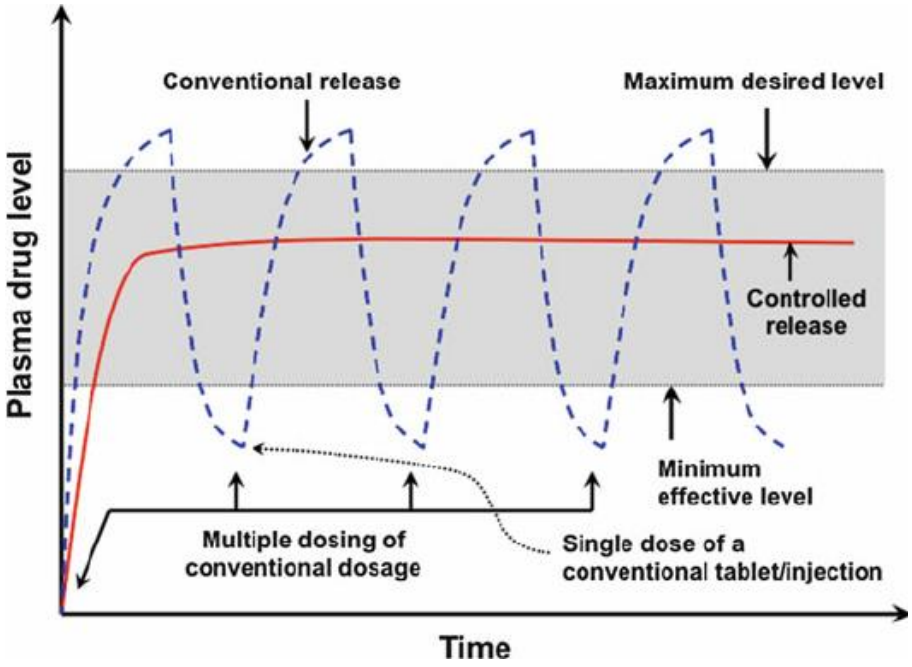

Figure 1.1: Drug administration.

For those reasons, DDS strategy involves an interdisciplinary research, involving polymer science, pharmaceutics, bioconjugate chemistry and molecular biology. The result presents many advantages:

- Maintenance of hematic concentrations within therapeutic levels for an extended period of time;
- Drug protection from hostile environment;
- Reduction of collateral effect, due to initial over dosing;
- Selectivity towards target tissue;
- Reduced number of administrations.

For those benefits, that correspond to a significant improvement of traditional active compound administration, drug delivery is a very appreciated research field and a lot of effort is done to develop new solution of the control enhancement of pharmaceuticals compounds. Equally important, those advances are also more attractive for the relative low costs of drug delivery formulations or devices with respect to development of new advanced drugs (Tiwari et al. 2012).

1.1.1 Drug release mechanisms

The mechanisms involved in drug release are mainly four:

- *Diffusion controlled systems*

  Delivery is driven by concentration gradient existing between the inside and outside of device. The parameter useful to describe the tendency of the drug to move outward is the diffusion coefficient *D*, dependent from steric hindrance, viscosity and temperature.

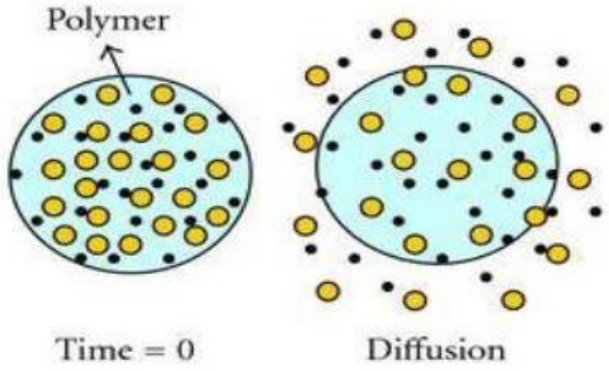

Figure 1.2: Diffusion controlled release mechanism.

There are basically two types of diffusion devices: a drug bulk surrounded by a polymeric barrier and a monolithic polymeric system in which drug is uniformly dispersed into the matrix.

It's easier to produce monolithic systems, but it's difficult to obtain zero order kinetics, because gradient is decreasing during time, on contrary of reservoir devices.

- *Swelling controlled systems*

  This mechanism is related to the release of the drug following the hydration of systems. These devices are designed using water as the main agent controlling the drug release: the active compound cannot diffuse out of device without water molecules diffusing in. Swelling controlled systems are based on hydrophilic polymers: in dry state, polymeric network is dense and the mobility of macromolecules is very much restricted. Upon contact with water, the polymer chain "relax", assuming an elongated conformation increasing the mesh size. The consequence is that the macromolecules are now free to diffuse, following a classic Fickian behaviour, and the system volume is significantly increased.

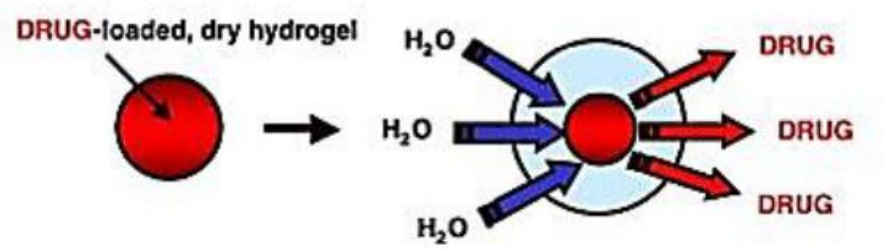

Figure 1.3: Releasing mechanism controlled by swelling.

- *Bio-erosion controlled systems*

  This mechanism is based on the erosion of polymeric structure and consists on the selective leakage of some chemical bonds in polymeric structure up to its reduction to oligomer or monomer that human body is able to eliminate. This process is the result of the exposure to chemicals (water) or biologicals (enzymes).

Hydrolysis is the most frequent reaction exploited to break polymeric chain: a long chain is divided into shorter one by the addiction of a water molecule.

$$P_{n+m} + H_2O \rightarrow P_n + P_m$$

If the diffusion of water into polymeric matrix is faster than the de-polymerization kinetics, a uniform degradation occurs onto the entire matrix, and it's known as "bulk degradation". On the way opposite, if erosion dynamics are faster than water diffusion, superficial degradation takes place.

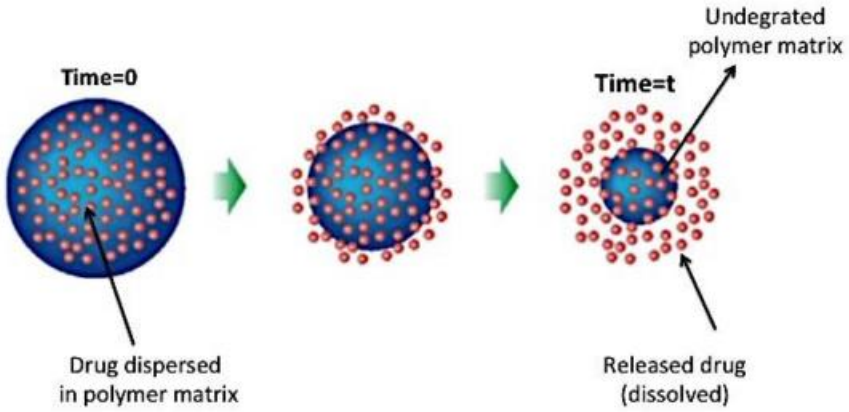

Figure 1.4:Drug release due to erosion phenomenon.

- *Osmosis controlled systems*

  Those devices are called "elementary osmotic pumps" and they are constituted by a central core osmotically active (containing drug) surrounded by a semi-permeable polymeric membrane with a hole. When the pumps are immersed into water, this is able to diffuse through polymeric membrane, dissolving the drug and pushing it out from the hole, thanks to the augmented pressure brought by the volumetric increase. This device can work properly just with sufficiently water-soluble active principles.

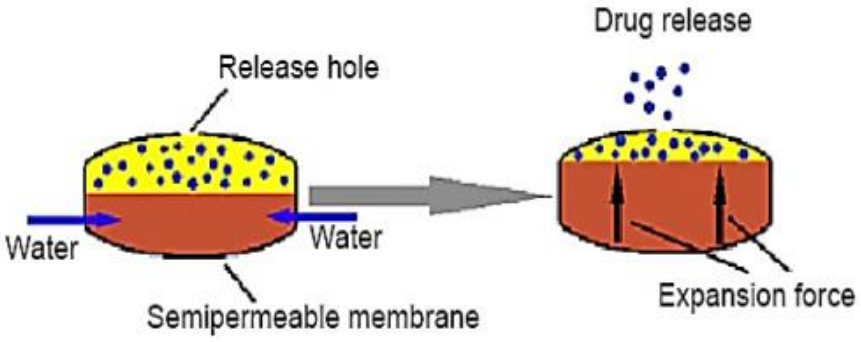

Figure 1.5: Drug release due to osmotic pressure.

The former three mechanisms can be applied to polymeric carriers, that are gaining interest due to their functionalisation possibility and targeting capacity towards specific target cells, in addition to low costs and their suitable physico-chemical properties.

1.1.2 Polymeric drug delivery systems

Research in area of controlled drug delivery systems has obtained great interest due to their advantages in terms of safety, efficacy and patient convenience avoiding risk of surgery. In addition, drug delivery devices exist in many forms and can be administered via different routes, depending on disease, the desired effect and the availability of product (Tiwari et al. 2012).

Anatomical routes
Oral
Parenteral • Subcutaneous injection • Intramuscular injection • Intravenous injection • Intra-arterial injection
Transmucosal
Transnasal
Pulmonary (by inhalation)
Transdermal drug delivery
Intraosseous infusion

Table 1.1: Anatomical routes for drug delivery.

Drug delivery systems, lipid- or polymer-based nanoparticles can be designed to improve pharmacological and therapeutic properties of drugs. Synthetic polymers were the earlier biomaterial used for drug delivery purposes, due to their easy large-scale production and highly tunable properties. Both of them contribute to the large number of formulations present in literature.

Polymeric carriers as drug delivery devices are able to increase specificity of release of drug inside the human body, minimizing the systemic distribution and increasing its therapeutic activity. Once this has been accomplished, he carrier is degraded and eliminated shortly. We can divide polymers into three groups (Davis 2000):

1. Nondegradable polymers: they are stable in biological systems and for this reason are most used as components of implantable devices for drug delivery.
2. Drug-conjugate polymers: drug is now attached to water-soluble polymer carrier by a cleavable bond. These polymers are less accessible to heathy tissues in comparison with diseased ones and can be used to convey drug to the target via systemic administration or by implanting them directly in the site of action.
3. Biodegradable polymers: those components can be degraded in biological environment to non-toxic products, that are easily eliminated from the body.

Commonly, polymers can be used both as synthetic or derived from natural sources.

In contraposition to the advantages of naturally derived polymers, synthetic polymers offered wider scope to design and control the characteristics of the material. Moreover, the possibility to reduce the allergenic risks using a completely artificial biocompatible material is evident.

On the other side, the use of natural polymers has been gaining widespread attention owing to their favourable attributes of biodegradability, low toxicity, low manufacture and disposal costs.

Moreover, they offer a wide range of advantages for tissue engineering applications, such as biological signaling, cell adhesion and cell responsive degradation and re-modelling (Rossi, Perale, and Masi 2016).

Generally, desirable characteristics for polymeric systems used in drug delivery are to get a minimal tissue reaction after implantation, high polymeric purity and reproducibility and a reliable drug-release profile.

1.1.2.1 Synthetic polymers

Among synthetic polymers, polyesters are the most interesting from an industrial point of view: they contain an aliphatic ester bond in their backbone. Although theoretically all of them are degradable, only polyesters with reasonably short chains can be used as degradable polymers for biomedical applications. They are mildly hydrophobic and esters bond stability causes them to undergo bulk erosion (Yu et al. 2011). The main returns of those polymers are high purity, easy process, good mechanical properties and their biodegradability, that is the ability of organism to degrade products of polymers hydrolyzation, which can be resorbed through normal metabolic pathways.

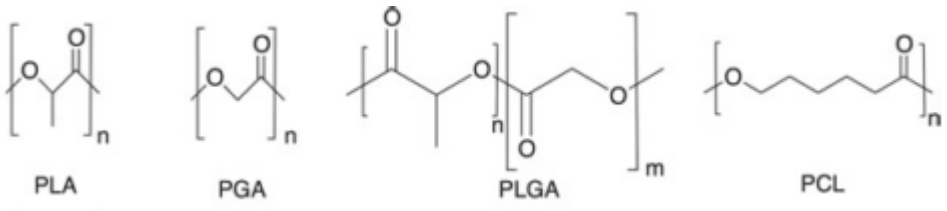

Figure 1.6: Polyesters for drug delivery.

Another category of polymers very exploited in tissue engineering due to their hydrophilic nature and controllable, reproducible chemistry are polyethers. Those compounds, like poly(ethylene-glycol) (PEG), are very interesting due to their versatility, which allows to control molecular weight, cross-linking density, degradation rate and mechanical strength, that can be seen as a good trade-off between cytocompatibility and mechanical requirements.

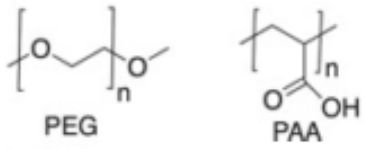

Figure 1.7: Polyether and polyacrylate used in drug delivery.

Moreover, polyamides and acrylates are very investigated as proper materials for biomedical devices, like scaffolds for tissue engineering and controlled drug delivery in different targeted tissues.

1.1.2.2 Natural polymers

Polysaccharides are the most frequently employed natural polymers in biomedical applications. Although they can present often not negligible content of allergens and contaminants, they have some excellent characteristics that lead to their wide exploitations, like non-toxicity, stability to pH variations and the possibility to be functionalized both biologically and chemically.

One of the most interesting polymers for tissue engineering application is the chitosan, or the *D*-glucosamine and *N*-acetyl-*D*-glucosamine copolymer, a hydrophilic, biodegradable and non-toxic compound that allows cell adhesion, proliferation and differentiation, inducing minimal foreign body reaction.

Also, Hyaluronic acid has demonstrated to be appealing in biomedical devices because of its capacity to absorb and retain water, that lead to confer extraordinary viscoelastic properties to material. Along with this biomacromolecule, collagen is another natural polypeptide, already present inside human body, which has valuable properties promoting cell proliferation and differentiation.

Finally, alginate represents a useful material for the creation of hydrogels, because of its physico-chemical properties that allow it to cross-link under very mild conditions, at low temperature and without any organic solvent.

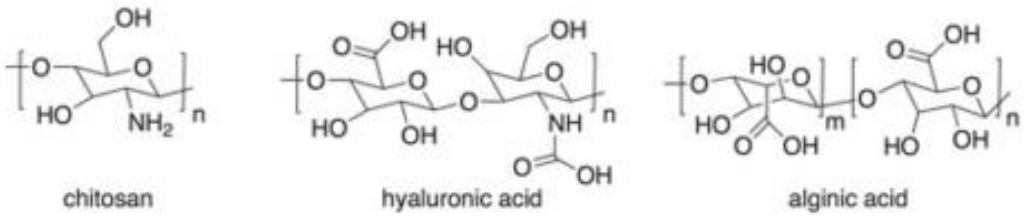

Figure 1.8: Polysaccharides used in drug delivery.

Focusing on nanoparticle systems, polymers are extensively used for their physical, biological and chemical properties, that can be easily modified to meet specific applications. Another advantage is that they're convenient materials for the manufacture of countless and varied molecular design, that can be integrated into unique nanoparticle constructs. Several polymers have been approved by the U.S. Food and Drug Administration (FDA) for human applications, that should be adaptable, in terms of non-toxicity and non-antigenicity, biocompatible and biodegradable. Some of the most promising are polylactides (PLA), poly(lactide-co-glycolides) (PLGA), polycaprolactones, poly(methyl methacrylate), poly(ethylene glycol) (PEG), among synthetics. Natural polymers wildly used in this application are Sodium alginate, Chitosan and Albumin (Nagavarma et al. 2012).

1.1.3 Drug administration routes

The pharmacologic therapy must take into account also the constraints linked to administration ways, that should be selected according to the type of injury or disease and that can now be improved using drug delivery devices.

- *Transdermal or topical*

  Quite novel system to deliver drugs that assures local effect, although absorption is quite slow because it occurs through the skin, that is a quite impenetrable barrier, in particular in the outermost layer made of lipids and keratin. An occlusive dressing may be used to improve absorption and transdermal patches can provide a prolong and controlled drug delivery, also with the presence of some chemical enhancers, that improve drug absorption. In addition, it is also possible to modifying chemically the drug, to make it more lipophilic to enable it to pass through the skin. Drug absorption will vary by site of administration, skin condition, age and gender (Bertoldo 2015).

- *Oral*

  This is the most classic administration route of drug and one of the major goal is to achieve a constant release as it passes through the stomach and gastrointestinal tract and to reduce the number of pills needed. An improvement in this direction should be binding charged drugs to ion-exchange resins that can be coated with semipermeable membranes. Another possibility is to use erodible polymers where the outer layer acts as a diffusion layer, or again to use a microcapsule to sustain and tune release rates.

- *Injectable hydrogel*

  In the field of injectable systems, hydrogels are becoming more and more important for biomedical applications. They are three-dimensional networks of hydrophilic polymers held together by covalent bonds or other cohesive forces.

They can retain a large amount of solvent, generally water, passing from a dry, glassy state to a swelled elastic structure.

Those scaffolds are studied to be degraded in physiological environment, minimizing the risk of surgical procedure due to their capability to form a 3D network in situ after injection.

- *Nanosystems*

  They are a novel system for drug administration and appear more suitable than other tools because they're characterized by more versatility in term of size, surface charge, surface modification and hydrophobicity. In addition, they can enter smaller capillaries, cross different biological barriers, being up taken easily from cells. A wide variety of systems have been developed, each with their unique advantages and disadvantages.

  They can be divided into:

  1. Polymeric/magnetic nanoparticles;
  2. Drug-polymer conjugates;
  3. Solid lipid nanoparticles;
  4. Liposomes;
  5. Micelles;
  6. Metal nanoparticles;
  7. Carbon nanomaterials.

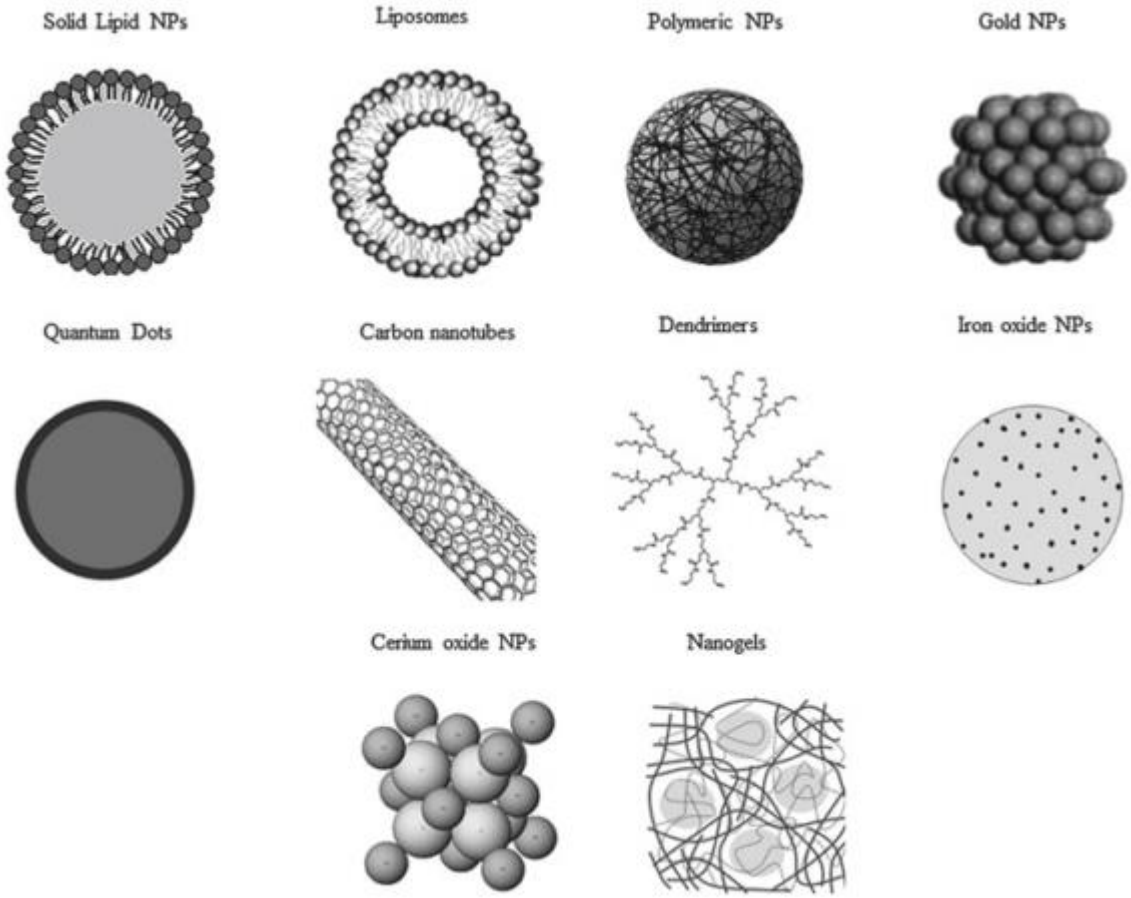

Figure 1.9: Overview of nanosystems methods for drug delivery.

1.1.3.1 Nanoparticles

Among all possible nanosystems, we focus our attention on polymeric nanoparticles (NPs), that in the last two decades have been applied as therapeutic and diagnostic agents for the treatment of a vast variety of diseases.

Many advantages have been recognised for nanoparticles devices, that improve solubility of poorly water-soluble compounds, prolong the half-life of drug systemic circulation, release drugs at a sustained rate or in an environmentally responsive manner and thus lowers the frequency of administration, deliver drug in a target way to minimize systemic side effects (Darling-Hammond 2000).

After pharmacological load release, nanovectors should be metabolized and cleared out without bioaccumulating.

The key factors in drug delivery process are the particle size and the size distribution, so the nanoparticles characterisation is very important to determine their systemic distribution and internalization mechanism, their toxicity and targeting ability. In addition, they can influence nanoparticle stability and drug loading and release.

Indeed, smaller particles have higher surface-volume ratio and the drug molecules majority will be found near the surface, causing a faster release.

It has been demonstrated that NPs smaller than 500 nm are internalized by endocytosis mechanism, while bigger ones by phagocytosis (Rejman et al. 2004). NPs cellular uptake is one of their most interesting characteristics, because of their small dimensions and high mobility.

On the other side, NPs with diameter of 30-40 nm are easily cleavable from the kidney so they have small half-life time. At the same time particles with diameter bigger than 200-250 nm are eliminated by reticuloendothelial system (Bertoldo 2015).

Polymers used for NPs should present the same characteristic before mentioned, such as biocompatibility and biodegradability, along with stability in biological conditions.

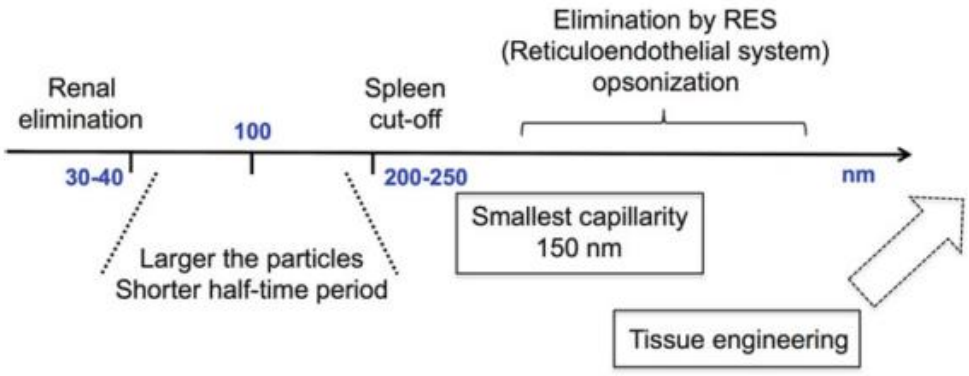

Figure 1.10: Optimal NPs diameter range.

Moreover, in case of nanoparticles application, they must have also low polydispersity, to be able to create uniform NPs dimensions. A great advantage is to have a high reproducibility in term of synthesis.

1.2 Spinal Cord Injury

The spinal cord is a tubular bundle of nervous tissue and supporting cells with 8-10 mm of average diameter; it extends from the medulla oblongata in the brainstem to the lumbar region of the vertebral column.

The ensemble of brain and spinal cord forms the Central Nervous System (CNS), that is the centre in charge of reception, elaboration and information transmission in all other body districts. Once an external stimulus is detected, is converted into an electrochemical signal, that is transported along the axons to the dorsal side of spinal cord toward the brain. Here, it's elaborated and the responsive motor signal goes back into the ventral side of spinal cord to the region that perceived the stimulus.

This makes spinal cord essential for health and very delicate. The so called spinal column is assigned to be a protection for the spinal cord: it's made by vertebrae, or hollow bones, that are stacked one on the other and splitted by cartilage disks to form a column in which spinal cord runs.

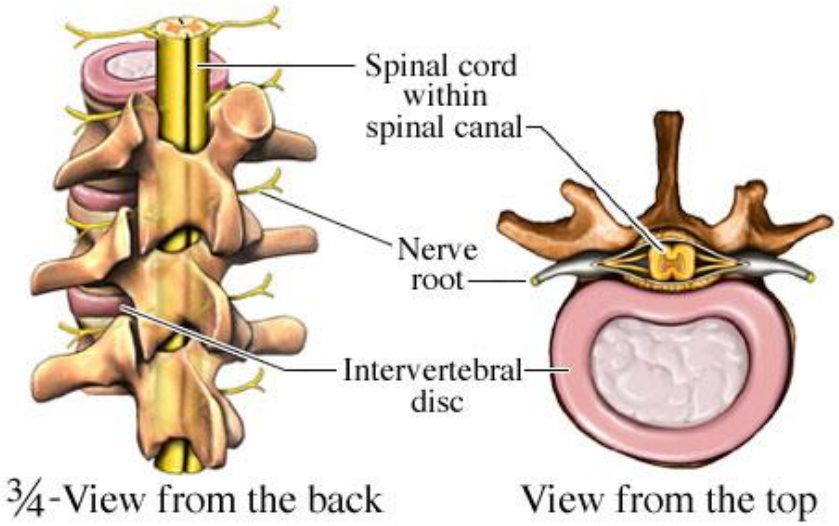

Figure 1.11: Spinal column anatomy.

With a protection aim, there are also three membranes, called meninges, that covers the spinal cord. The external is the *dura mater*, due to its strength, followed by the intermediate *arachnoid*, made by thin and web-like filaments. Finally, the inner one is the *pia mater*. Between the arachnoid and pia mater there's a space filled with cerebrospinal liquid and some blood vessels, that in case of trauma lead to haemorrhaging phenomena.

The spinal column is divided into five regions: cervical, thoracic, lumbar, sacral and coccygeal.

A spinal cord injury (SCI) is a damage to the spinal cord that results in a severe sequela, such as intense pain and progressive neurological damage and changes in its functions, either temporary or permanent. Those changes translate into loss of muscle function, sensation or autonomic function in parts of the body served by the spinal cord below the level of lesion. Injuries can occur at any level of spinal cord.

In the majority of cases, the damage results from a physical trauma, such as car accidents, gunshot, falls or sport injuries, but it can also result from non-traumatic causes such as infection, insufficient blood flow and tumors.

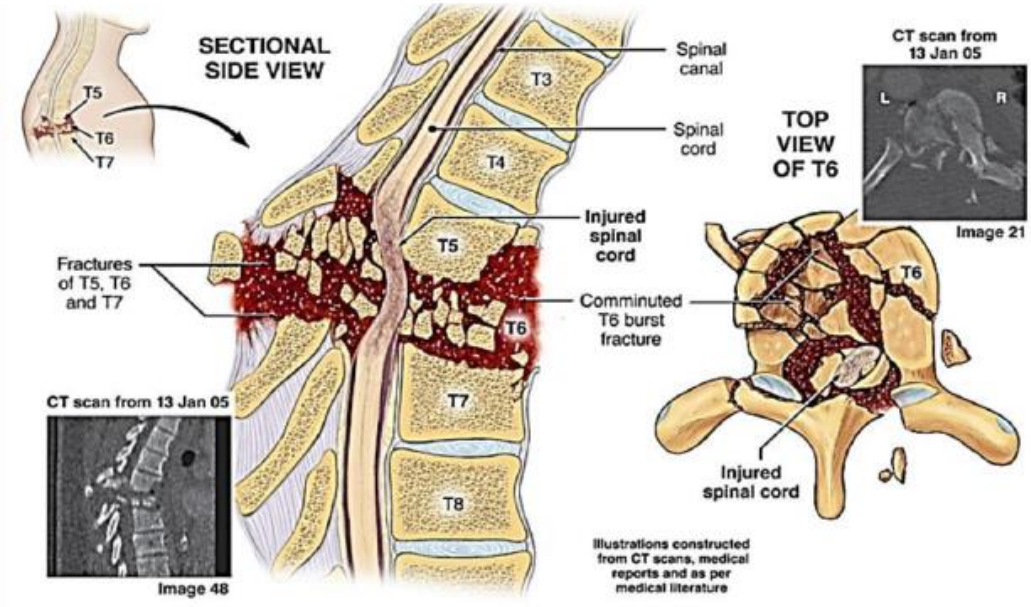

Figure 1.12: Spinal cord injury at vertebrae T5, T6 and T7.

Due to the violence of trauma, a high percentage of people victim of SCI dies before hospitalization, but who survives the trauma has a lower life expectancy with respect to sane people, in dependence of the resulting condition (paraplegia or tetraplegia).

When the SCI occurs, the spinal cord swells and the effect have implications all over the organism. After days or weeks, the swelling decreases and some functioning may be regained, due to hematoma reabsorption and reduction, although the recovery possibility generally ends at about six months.

SCI can be divided into three phases, that differ in time and in mechanism involved (Zhou, He, and Ren 2014):

1. Primary phase: occurring from seconds to minutes after the traumatic event, it's typically restricted to the specific area of vertebral fracture and it's characterized by acute haemorrhage and ischemia, which serves as the starting point for the subsequent secondary mechanism of injury.

   In this case, the damage mechanism is characterized by direct destruction of spinal tissue, including the blood spinal cord barrier.
2. Secondary phase: is characterized by further destruction of neuronal and glial cell, that leads to a significant expansion of the injury site and allows paralysis to extent to adjacent spinal cord segment. It's a cascade of biochemical and cellular processes started from primary event and that may cause inflammation and a subsequent worsening of patient conditions. Indeed, the acute inflammation is characterized by microglia activation and immune cell infiltration (lymphocytes, neutrophils and macrophages) and by the release of inflammatory mediators, occurring just after few minutes after the injury. This leads to spread and exacerbation of tissue injury.

Microglia and macrophages are the key cellular players involved into inflammatory events, because they respond to traumatic insult by adopting an activated phenotype that persist until some weeks after the event, but promoting both injury and repair, depending on the different phenotype (Papa et al. 2013).

3. Chronic phase: when the inflammation diminishes the chronic phase succeeds and a scar forms around the injury that isolates and protects the damaged tissue, but obstacles nerve regeneration as well.

It's demonstrated that microglia are responsible for both destructive and regenerative response, as they are rapidly activated after a traumatic event and can assume two different phenotypes, activating a pro-inflammatory response (phenotype M1) or, alternatively, an anti-inflammatory response (phenotype M2).

M1 microglia originally respond to the injury and infection, acting as the first line of defence against invading pathogens. However, they also introduce neurotoxic mediators and often setup a vicious cycle between dying neurons and acute inflammation.

After the onset of classical activation, an anti-inflammatory and repairing phase is starting that leads to wound healing and tissue homeostasis. M2 are the major effector cells with the potential to dampen pro-inflammatory immune responses and promote the repair genes expression (Chincarini and Rigamonti 2016).

The therapeutic goal after SCI is to limit the tissue damage and to prevent neuronal and axonal death. Therefore, is important to comprehend the spinal cord acute inflammatory response, its time evolution and characteristics, not only to obstruct the negative effects, that cause a worsening of the initial damage, but event to preserve and possibly encourage the ones that promote regeneration and restoring.

In primary phase, or acute phase, the first possibility is to reduce the compression on the trauma region to protect it from more damage.

Indeed, the landlocked inflow of blood can suffocate the neuronal cells and cause tissue degeneration. Thus, a chirurgical decompression is recommended for a better prognosis. The principal method to realize such decompression is the spinal traction, followed by column stabilization. In this phase, the only pharmacological treatment that has shown some efficacy is methylprednisolone (MP), administered systemically during the first 8 h after SCI occurrence. However, the high doses needed to be effective have been demonstrated to produce side effects even worse than benefits, such as pneumonia, pulmonary embolism, sepsis and even death (Kim, Caldwell, and Bellamkonda 2009).

In chronical phase, various therapies are possible, both pharmacological and rehabilitative. Rehabilitation may be active, with patient's voluntary effort to improve motoric abilities, or passive, where movements are induced by physiotherapists or machines. On the other sides, drugs are used to control spastic movement and pain, to enhance bladder and bowel control (Bertoldo 2015).

In this view, a pharmacological approach, studied to modulate microglia/macrophage activation, would provide a better chance to interfere with the inflammatory event related to the expression of M1 (Papa et al. 2013). Furthermore, since toxicity of methylprednisolone can be related to high systemic dosage, an upgrading of drug delivery can lead to improve the clinical outcomes in the therapy of secondary injury (Kim, Caldwell, and Bellamkonda 2009).

1.2.1 Combination therapy: nanoparticles-hydrogel composites

One of the newest approaches for local and sustained methylprednisolone release onto the injured spinal cord tissue is to use biodegradable polymer-based nanoparticle.

This method has demonstrated lots of advantages with respect to the systemic administration (Kim, Caldwell, and Bellamkonda 2009):

- Better therapeutic effect;
- More efficient, targeted drug delivery to injury site: MP delivered through systemic administration is influenced by short pharmacokinetic half-life of drug, and for this reason this delivery method needs high-dosage of MP, which results in very negative side effects. Using polymeric nanocarriers, the dose on nanoparticle-encapsulates MP is much lower and this local delivery technique enhance therapeutic effect by increasing MP concentration levels just at target site.
- Potential adjustment of delivery rate and duration: since release profile from nanoparticles can be easily controlled by tuning the biodegradable polymeric composition, rate, amount and duration of delivery can be costumed.
- No need of surgery: nanoparticles can be stored as lyophilized powder and easily resuspended of embedded in hydrogel and locally delivered through injection onto le lesion site.

This final advantage of using nanoparticles is representative of a great field of research based on the definition of a combined therapy, that is the application of multiple therapies to treat one single disease: multifunctional therapies for SCI treatment is directed to counteract multiple injury mechanism, combining both neuroprotective and neuroregenerative agents, guarantying different controlled drug delivery kinetics for the two active compounds.

Indeed, hydrogels represent a class of soft materials very interesting in biomedical field due to their physicochemical properties, compatible with biological tissues. They are physically or chemically cross-linked three-dimensional network, that can retain considerable amounts of water, but they cannot load hydrophobic drugs, due to their very nature.

Furthermore, drug loading can be uneven inside the network and the pore size can often causes uncontrolled drug release (Hoare and Kohane 2008).

On the other side, NPs, thanks to their versatility in terms of size, surface potential and hydrophilic/lipophilic characteristics, lead relevant advantages in drug delivery by increasing selectivity of drugs and by controlling their release during time. However, if injected by themselves, they often leave the zone as they're not confined and easily extraversate into the circulatory torrent, migrating all over the body to liver and spleen or toward an uncertain faith (Rossi et al. 2013).

For those reasons, recently, the research trend is focused on the advances in developing nanostructured hydrogels, or composites of nanoparticles embedded in hydrogel matrix. The interaction of these nanosystems with the polymeric chains of hydrogel structure results in the peculiar properties of nanocomposite, absent in the individual components (Thoniyot et al. 2015). In fact, nanoparticle addition may reinforce the starting hydrogel and provide the composite the responsiveness to external stimuli, such as mechanical, thermal, magnetic or electric depending on the nanoparticle nature, and improved loading capability.

On the other hand, introducing nanoparticles into a hydrophilic support is a benefit for the release of drugs *in situ*.

Thus, we can design a composite support for drug delivery able to retain and administer both hydrophilic (easily loaded into hydrogel network) and lipophilic drugs (contained into polymeric nanoparticles), with different kinetic of release, tunable controlling the hydrogel and nanoparticles compositions (Biondi et al. 2015).

There are three different hydrogel-nanoparticles designs that can be proposed (Thoniyot et al. 2015):

a. Micro- or nano-sized hydrogel particles stabilizing inorganic or polymeric nanoparticles;
b. Nanoparticles non-covalently immobilized in a hydrogel;
c. Nanoparticles covalently immobilized in hydrogel matrix.

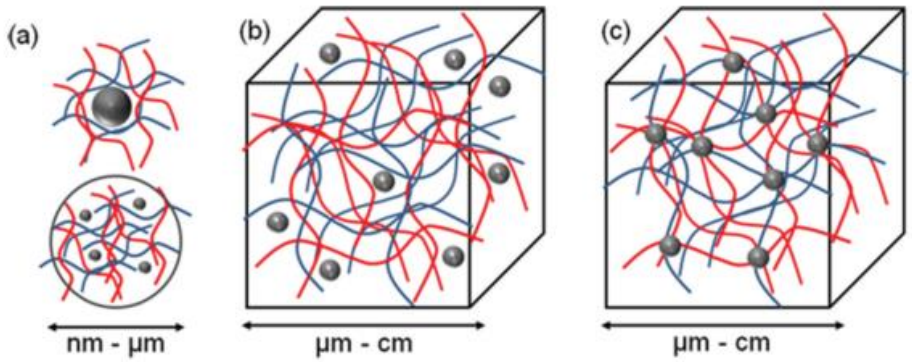

Figure 1.13: Different structural design for hydrogel-nanoparticles composite material.

2 Colloidal systems

Colloidal systems are mixtures of two or more components where we can identify a so called *dispersed phase*, made by suspended particles, spread throughout a *continuous medium*. Both dispersed and dispersing substances can be in solid, liquid or gaseous form.

Colloidal particles exist in a dispersed state and they are intermediate in size between molecules and the smallest piece visible under an optical microscope, generally in the range of 1 nm to 1 μm (Sarquis 1980). Thus, they create a system that lies in between a proper solution (homogeneous mixture made by a single phase) and a suspension (heterogeneous mixture where suspended particles are clearly visible and tends to settle in time).

For this reason, colloids present characteristics different from both solutions and suspensions (Kotz et al. 2006):

- Colloidal particles have huge molecular mass, so that generally they deal with macromolecules, such polymers, proteins or others;
- Thanks to their dimensions, they can scatter light, phenomenon known as Tyndall effect. By virtue of this property, it's possible to determine commercially size and density of particles in a colloidal matter;
- Always because of their dimensions, they are characterized by Brownian movement, a random and independent migration that tends to uniformly fill the entire volume of the dispersant medium.
- Colloidal particles have such dimensions that their bulk properties are not so important with respect to surface characteristics, which are actually describing those systems.

The more finely a material is divided and its surface area increases, the grater the proportion of atoms/molecules found at the surface rather than in bulk material.

Colloidal systems are classified, accordingly to the nature of interaction between the dispersed phase and the continuous medium, as lyophilic or lyophobic. If the dispersant is water, it's possible to define hydrophilic or hydrophobic colloids.

Hydrophobic colloids are defined as colloidal system in which the dispersed phase is not interacting with water, such as hydrophobic polymers. They are thermodynamically unstable and represent a suspension of colloidal size aggregates difficult to prepare, due to their instability. Two methods are possible: *dispersion* of large particles that are mechanically grounded to obtain colloidal sizes, or *condensation* of smaller particles which aggregate until they are large enough to be considered colloids.

On the other side, hydrophilic colloids involve hydrophilic molecules that interacts with water through some functional groups (such as -OH or $-NH_2$) obtaining hydrogen bond, able to stabilize the system. The preparation of those systems is much easier than hydrophobic because it involves just heating.

Those systems are intrinsically unstable and tends to sediment, creating two distinct phases completely separated. That's because colloidal particles collide with each other due to Brownian motion, convection and gravity forces. Such motions are contrasted by viscous forces, but their velocity could be enough to promote flocculation and also coagulation, destabilizing colloids (Sarquis 1980).

Indeed, looking to the thermodynamic of the problem, at constant temperature, a system tends to alter spontaneously in order to reduce its free energy:

$$\Delta G = \gamma_{SL} \Delta A$$

Equation 2.1: Free Gibbs energy variation of a colloidal system.

ΔG diminishes either by reduction of the interfacial tension between particle suspended and the liquid medium γ_{SL} or by a decreasing of the interface area.

Thus, without adding any surfactant that could act to reduce γ_{SL}, the system spontaneously tends to become coarser, to flocculate or coagulate.

Colloids of non-stabilized particles flocculate rapidly, as a consequence of long-range attractive forces, known also as London dispersion forces. If we model the systems as two spheres each of radius a, at a distance H_o, the attractive potential energy results as:

$$\Delta G_{att} \equiv V_{att} = \frac{A'a}{12H_0}$$

Equation 2.2: Attraction potential.

Where A' is the effective Hamaker constant, that gives an idea of the relative strength of the attractive phenomenon.

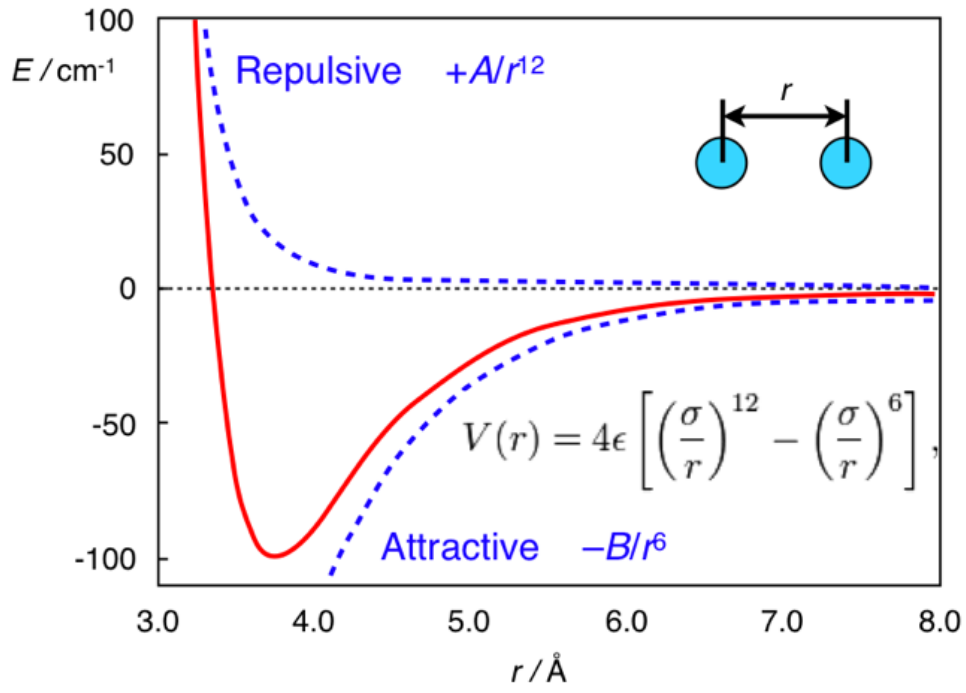

Figure 2.1: Intermolecular binding potential.

Actually, attraction between molecules occurs just until a certain distance after which a rapid increase of potential energy arises due to the repulsive interaction of electronic clouds, that cannot overlap (Napper 2006).

So, the total potential energy results to have a minimum point that correspond to an equilibrium state, where the attractive and repulsive forces balance.

According to the kind of repulsion force, two mechanisms of colloidal stabilization take place:

- *Electrostatic stabilization*

  In this mechanism, the attraction due to van Der Waals forces is counterbalanced by the repulsive Coulomb forces acting between negative or positive charged

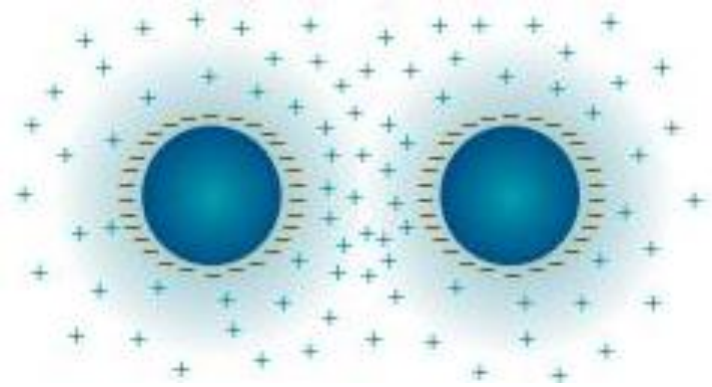

Figure 2.2: Electrostatic stabilisation of negatively charged nanoparticles.

  polymeric particles.

  When two charged bodies approach each other, the corresponding double layers overlap and local ion concentration increases, obtaining repulsion.

  In this case, the electrolyte concentration inside the solvent is crucial because it affects strongly the double layer thickness on the particle, hindering the stabilization.

- *Steric stabilization*

  It's obtained by adsorbed/grafted polymer molecules on particle surface and the stabilization mechanism is related to polymer thermodynamics in solution and to the change of free-energy due to overlap between polymer layers. In this case we have two main contributions to stabilization: mixing and elastic.

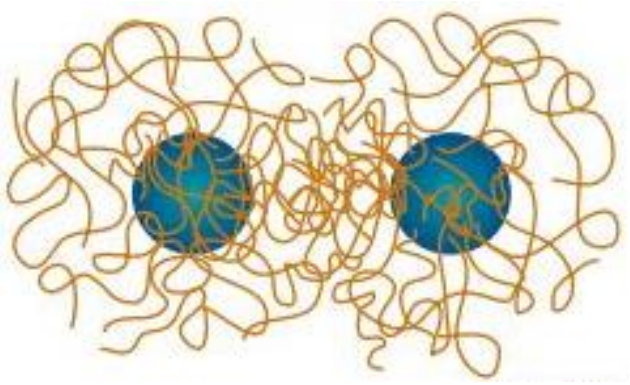

Figure 2.3: Steric stabilization of nanoparticles.

The first one is related to the interaction between polymers and dispersant medium: a polymeric chain in a solvent is arranged as a random coil. When two particles approaches, the corresponding free energy change is directly related to the Hildebrand interaction parameter. If the affinity between the polymer and the solvent is low (poor solvent), the interpenetration of polymers is favoured, because they tend to stabilize each other. On the other side, if the interaction between polymer and solvent is high (good solvent), the interpenetration is not thermodynamically favoured and the steric stabilization is achieved. Thus, the choice of polymer-solvent pair is essential. The consequent repulsive potential variation is:

$$\Delta G_{rep}^{E} = \pi a \frac{64 n^{0} kT}{k^{2}} Z^{2} \exp(-kH)$$

Equation 2.3: Electrostatic repulsion potential.

The elastic contribution, differently from the former, is generally always positive, because the interpenetration limits chain configuration possibility, thus reducing the configurational entropy.

The total interaction, for good solvents, so that interpenetration is not favoured, is always positive:

$$\Delta G_{rep}^{S} = \Delta G_{mix} + \Delta G_{el}$$

Equation 2.4: Steric repulsion potential.

Obviously, when possible, the mixed stabilization is the most versatile solution, that combines the positive effects of both steric and electrostatic mechanisms.

Consequently, the total potential energy of interaction between two particles depending on their distance is evaluated as addition of individual contributions.

$$\Delta G_T \equiv V_T = \Delta G_{att} + \Delta G_{rep}^{E} + \Delta G_{rep}^{S}$$

Equation 2.5: Total potential energy.

Graphically, the energy barrier that prevent flocculation or coagulation is clearly evident, and behaves like an activation energy for the coagulation of the system (Napper 2006) (Figure 2.4 and 2.5).

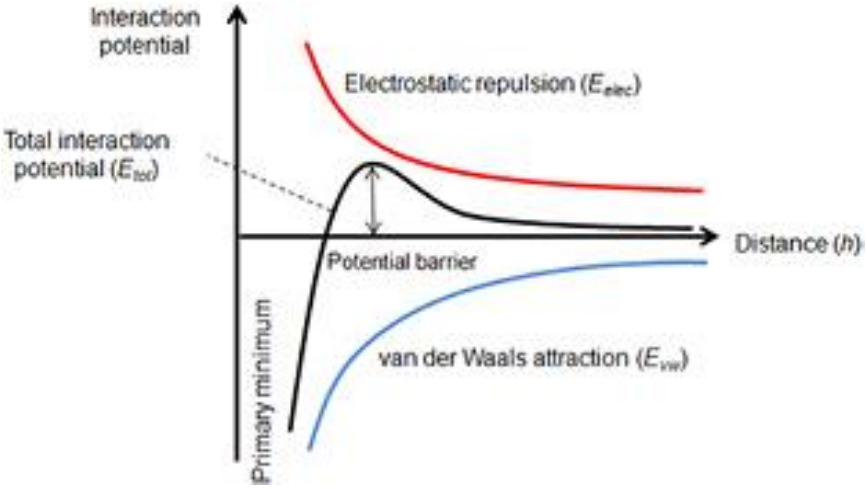

Figure 2.4: Electrostatic stabilization potential.

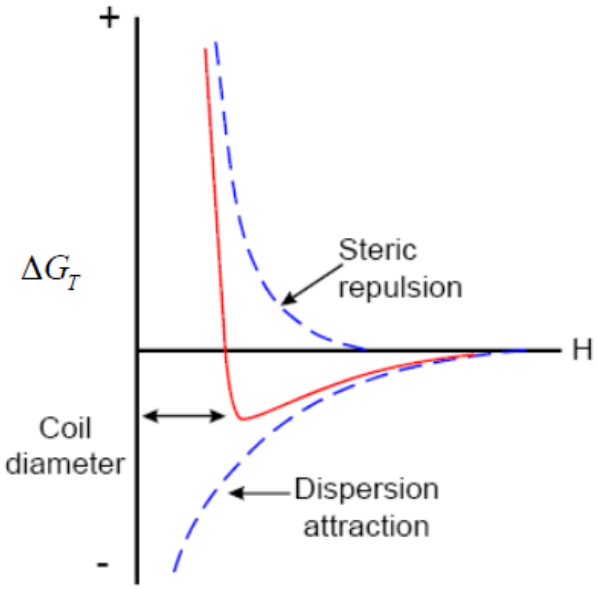

Figure 2.5: Steric stabilization potential.

Colloidal systems are generally classified according to the state of dispersed substance and the dispersing medium, as reported in table 2.1.

Among all the colloids, one of the most promising system in biomedical field is represented by polymer colloid, usually defined as a dispersion of submicron polymer particles in a liquid (typically aqueous) medium.

The application areas for polymeric colloids are different, and in medicine and biotechnology polymeric nanoparticles and hydrogels are assuming more and more importance (Daniels, Sudol, and El-aasser 2001).

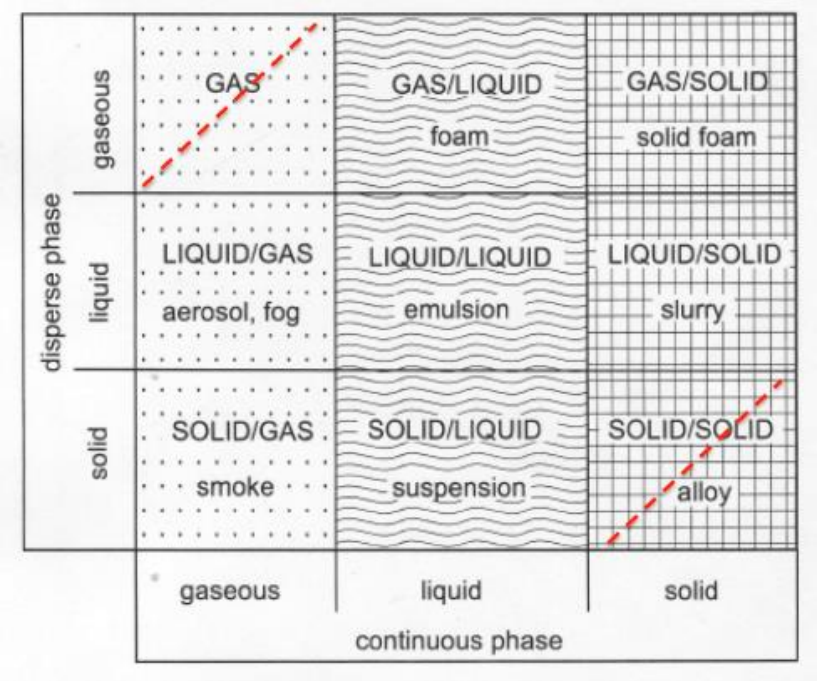

Table 2.1: Colloidal systems.

2.1 Nanoparticles

Polymeric nanoparticles (NPs) have attracted the interest of many research groups and have been used in an increasing number of fields during the last decades because of their stability when in contact with biological fluids, higher than other colloidal carriers'. They have been used as a physical approach to improve the pharmacokinetic and pharmacodynamic properties of various types of drug molecules, although they are simply particulate dispersions of solids with size in range of 10-1000 nm.

NPs are prepared from biocompatible and biodegradable polymers, as already said. Depending on the method of preparation, nanoparticles, nanospheres or nanocapsules can be obtained.

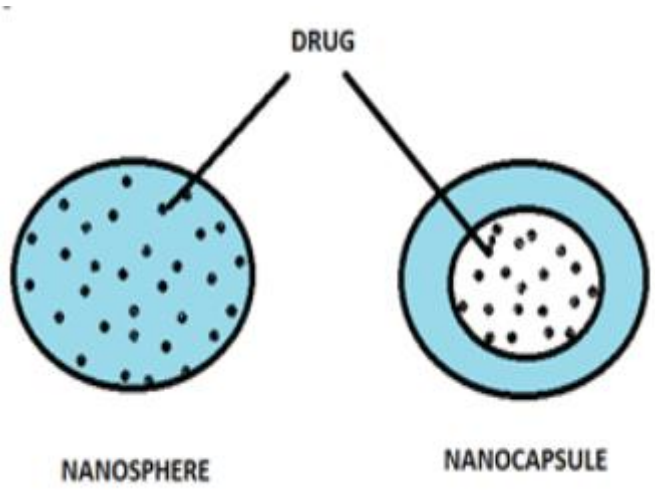

Figure 2.6: Difference between nanosphere and nanocapsule.

Nanocapsules are systems in which drug is confined into a cavity surrounded by a unique polymer membrane. On the other side, nanospheres are matrix systems in which the drug is physically and uniformly dispersed.

Several methods can be applied for the preparation of NPs and are classified according to whether the particle formation involves polymerization reaction or nanoparticles from directly form a macromolecule of preformed polymer (Nagavarma et al. 2012).

Methods based on dispersion of preformed polymers:

a) Solvent evaporation;
b) Nanoprecipitation;
c) Emulsification/solvent diffusion;
d) Salting out;
e) Dialysis;
f) Supercritical fluid technology.

Methods based on the polymerization of monomers:

a) Emulsion;
b) Mini/micro emulsion;
c) Interfacial polymerization;
d) Controlled/living radical polymerization.

In this work, we focus the attention on the former class of nanoparticles, although the selection of appropriate method for their preparation depends on the physicochemical characteristics of polymer and drug to be loaded.

Solvent evaporation

First method for the preparation of NPs and one of the most applied, solvent evaporation involves two steps.

The first one requires the emulsification of the polymer solution, containing drug, into an aqueous phase. Generally, the polymeric solution is obtained exploiting volatile solvents, such as dichloromethane or ethyl acetate, which has a better toxicological profile.

The emulsion is then converted into nanoparticle suspension into the second step, when the polymeric solvent is evaporated and the polymer is allowed to diffuse through the continuous phase of the emulsion.

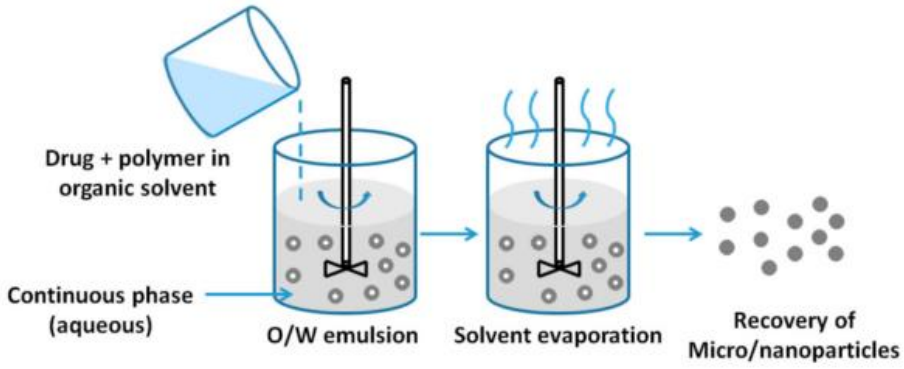

Figure 2.7: Solvent evaporation method.

The nanoparticles are collected by ultracentrifugation with washing stages with distilled water to remove additives, like surfactants, and lyophilized for storage. This method uses high-speed homogenization or ultrasonication, followed by solvent evaporation in continuous magnetic stirring. Particle size was found to be influenced by the type and concentration of stabilizer, homogenizer speed and polymer concentration (Pal et al. 2011).

Nanoprecipitation

It is also known as solvent displacement method: it involves the precipitation of a preformed polymer from an organic solution and the diffusion of the organic solvent in the aqueous medium in presence or absence of surfactant. Thus, the principle of this method is the Marangoni's effect. Polymer and drug are dissolved into a semipolar water miscible solvent, such as acetone or ethanol.

The solution is then injected drop-wise, with constant, defined rate, into an aqueous solution containing the stabilizer, under magnetic stirring. The solvent is then removed under reduced pressure.

The main advantage for this solution is that formation of NPs is instantaneous and needs only one step, so that it turns to be a rapid and easy operation. The rate of addition of the organic phase into the aqueous one is affecting the particle size: if it increases, both particle size and drug entrapment diminish (Fessi et al. 1989). Particle sizes have very narrow distribution because of the absence of shearing stresses, but this method is used mostly for hydrophobic drugs entrapment (Galindo-Rodriguez et al. 2004).

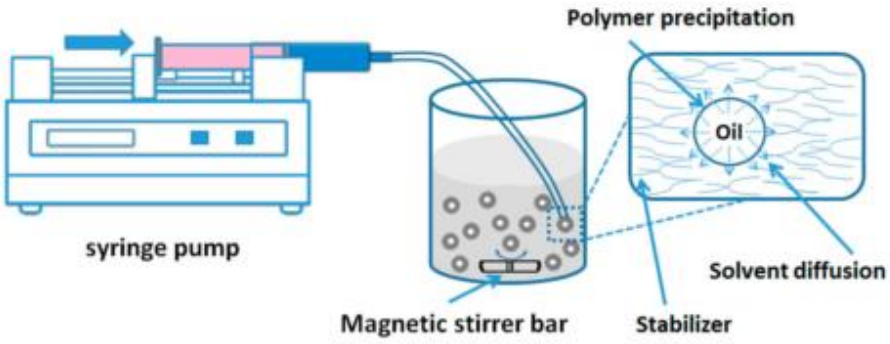

Figure 2.8: Nanoprecipitation method.

Emulsification/solvent diffusion

Interesting technique for the retainment of hydrophilic drugs. The encapsulating polymer is dissolved in a partially water-soluble solvent (propylene carbonate, benzyl alcohol) and saturated with water to ensure the initial thermodynamic equilibrium of both liquids. To force the precipitation of polymer and the subsequent formation of nanoparticles, it is necessary to promote the diffusion of the solvent of the dispersed phase by a dilution with an excess of water.

Thus, the polymer-water saturated solvent phase is emulsified in an aqueous solution containing stabilizer, leading to solvent diffusion and the formation of nanoparticles.

Finally, the solvent is removed by filtration or evaporation. This technique presents some advantages: high encapsulation efficiency, no need of homogenisation, high reproducibility, simplicity and narrow size particle distribution. On the other side, high amount of water is needed and its elimination can be a not negligible cost (Nagavarma et al. 2012).

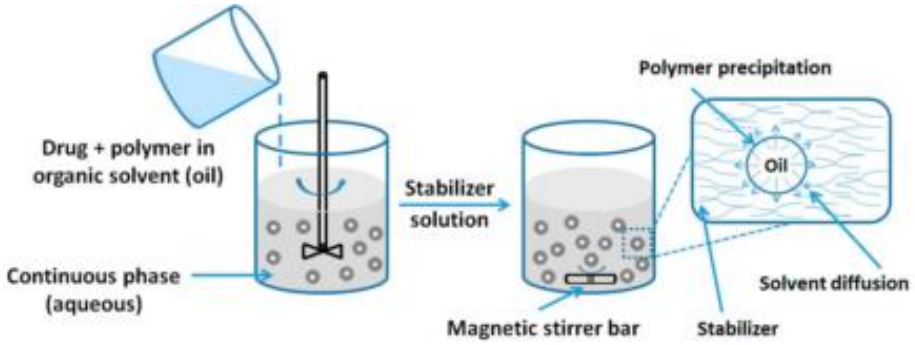

Figure 2.9: Emulsion diffusion method.

Salting out

This technique is based on the separation of water miscible solvent from aqueous solution via salting out effect (Reis and Neufeld 2006).

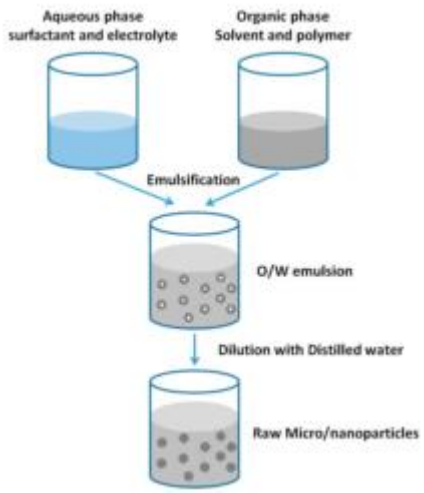

Figure 2.10: Salting out method.

Polymer is dissolved in organic solution, generally water-miscible, such as tetrahydrofuran (THF) or acetone.

On the other side, an aqueous solution of surfactant, saturated with electrolytes, that should be not soluble in organic phase, is prepared. The oil phase is then emulsified with aqueous solution, and the emulsion obtained is diluted with water to get a sufficient volume of aqueous solution to promote the organic solvent diffusion and so, inducing the formation of nanoparticles (Y. Wang et al. 2016).

Dialysis

It's a simple and effective method for the precipitation of small, narrow-distributed NPs. Polymer is dissolved into an organic solvent and placed inside a dialysis tube, with a proper molecular cut off. Dialysis is performed against a non-solvent miscible with the organic phase. The displacement of the solvent inside the membrane is followed by the progressive aggregation of polymer, which loss in solubility, forming a homogeneous suspension of nanoparticles.

Indeed, dialysis membrane is a semi permeable membrane that allows the passive transport of solvents to slow down the mixing of the polymer solution with a non-solvent.

The organic phase used in the preparation of the polymeric solution affects the morphology of the particles and their size distribution (Nagavarma et al. 2012).

Supercritical fluid technology

This is a NPs production option that is more environmentally and healthy safer, because it exploits supercritical fluids without any trace of organic solvents.

Supercritical fluids are defined as solvents with temperature above their critical one, at which the fluid remain in a single phase, regardless the pressure. The main used is the carbon dioxide, because of its non-toxicity, non-flammability and mild critical conditions, along with low price (Byrappa, Ohara, and Adschiri 2008). Two principles have been studied for the production of nanoparticles:

- **Rapid expansion of supercritical solution (RESS):** a polymer is dissolved into a supercritical fluid to form a solution, followed by the rapid expansion of the same across a capillary nozzle into ambient air. Nozzle temperature should be kept high to prevent precipitation in nozzle path. The result is a homogeneous nucleation and then the formation of well-dispersed nanoparticles. The concentration and the degree of saturation of the polymer have a considerable effect on the particle size and morphology. The main drawback is that the obtained product is microscaled rather than nanoscaled.
- **Rapid expansion of supercritical solution into liquid solvent (RESOLV):** possible development of the RESS, now the expansion is not done into ambient air but into a liquid solvent. The deal is that the liquid solvent apparently suppresses the particle growth in the expansion jet. In this way, it's possible to obtain mainly nanoscaled particles.

Therefore, depending on the mechanism of nanoparticle formation, it's possible to define several types of drug loading. In fact, the active principle can be carried by polymeric nanoparticles in different ways, that affects the final release. In many synthesis, it can be incorporated during the nanoparticle production, although the operative condition should be mild to not degrade the drug. Another possibility is to adsorb drug after the nanoparticle synthesis by incubating NPs in drug solution: a drug force is needed to allow entrapment into the polymeric particle, such as charge, pH or hydrophobicity (Allemann et al. 1993).

Finally, a chemical conjugation can be performed into the NPs, creating stable chemical bonds.

The drug encapsulation efficiency is one of the important parameter to identify in order to fully characterize the nanoparticle; it gives an idea about the drug percentage that is successfully entrapped/adsorbed into nanoparticles:

$$drug\ entrapment\ efficiency = \frac{drug\ mass\ loaded\ into\ NPs}{initial\ mass\ drug\ used} * 100$$

With some specific test, it's possible to know directly the entrapment efficiency measuring how much drug is loaded into the nanoparticles; the same thing can be obtained through the evaluation of the supernatant drug concentration residue.

Furthermore, the particle size distribution is also fundamental for biomedical applications and, along with surface charge and morphology, is the most important parameter to completely describe NPs. Indeed, the particle size affect the drug release: smaller the particle, larger is the surface area and faster is the drug delivery. On the contrary, drugs slowly diffuse inside larger particles. As a drawback, smaller particles tend to aggregate during storage and transportation of nanoparticle dispersion. Hence, there is a compromise between a small size and maximum stability of those nanosystems (Redhead, Davis, and Illum 2001).

Moreover, the nature and intensity of the surface charge is very important as it determines nanoparticles interaction with the biological environment. It can be evaluated through the zeta potential, which is an indirect measure of the surface charge. High zeta potential values, either positive or negative, should be achieved in order to ensure stability of the particles. It can also provide information about the nature of material encapsulated within the NPs or coated onto the surface (Pangi et al. 2003).

In this way, it's possible to identify the main properties of nanoparticles, predicting its behaviour inside biological environment.

2.2 Hydrogel

Hydrogels are unique hydrophilic networks of cross-linked polymers, both synthetic of natural, able to retain water up to thousand times their dry weight, maintaining a distinctive three-dimensional structure, characterized by different grade of entanglement of chains, depending on their formulations (Hoffman 2012).

One leading characteristic that make those systems so appealing in biomedical field is their ability to change in volume in response to small environmental variations, thanks to their swelling behaviour in contact with water (in swollen state, the mass fraction of water is much higher than the mass fraction of polymer), maintaining their physicochemical structure, although showing elevate elasticity, which gives them the ability to simulate biological microenvironment.

The peculiarity of hydrogels makes them suitable systems for drug, cell and growth factors carriers for therapeutic treatment of many diseases.

The solid reticulated matrix can be formed by chemical bonds of physical interaction, so that they can be classified as "reversible", when networks are held together by molecular entanglements and/or secondary forces, including ionic, H-bonding or hydrophobic forces, or "permanent", when they are covalently cross-linked.

The structure of an ideal hydrogel network is shown in figure 2.11.

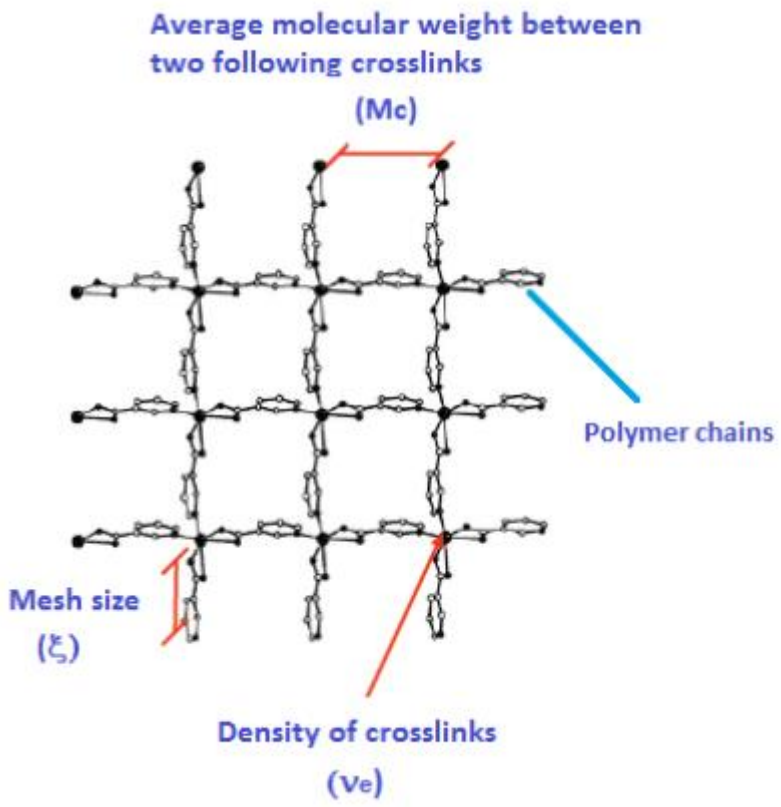

Figure 2.11: Network structure and important parameters of a hydrogel.

The peculiar variables that characterize structure and properties of a hydrogel are: mesh size, cross-linkage density, average molecular weight between two following cross-link points and volume, evaluated both in dry and swelling state.

Indeed, referring to the Flory-Rehner theory and equations (Ganji, Vasheghani-Farahani, and Vasheghani-Farahani 2010), the most important parameters that define the structure properties of hydrogel are:

- The polymer volume fraction in the swollen state $v_{2,\,s}$. It's defined as the ratio between the polymer volume V_p to the swollen gel volume V_g. It's also the reciprocal of the volumetric swollen ratio Q_V:

$$v_s = \frac{V_p}{V_g} = \frac{1}{Q_V}$$

Where Q_V can be defined as:

$$Q_V = \frac{\rho_p}{\rho_s}(Q_m - 1)$$

ρ_p is the density of the dry polymer and ρ_s the density of the solvent; Q_m represent the ratio between the weights of swollen polymer ($W_{swollen}$) and the dry polymer (W_{dry}).

$$Q_m = \frac{W_{swollen}}{W_{dry}}$$

- The effective molecular weight of the polymer chain between two following cross-linking points, designated as M_c. It's related to the degree of gel cross-linking X and the molecular weight of repeating monomeric units M_o.

$$M_c = \frac{M_0}{2X}$$

- The distance between the sequential points of cross-linking, ξ, which represent an estimate of space between macromolecular chains accessible for drug or cell diffusion. It can be calculated as:

$$\xi = v_s^{-\frac{1}{3}} * C\left(\frac{M_c}{M_0}\right)^{\frac{1}{2}}$$

where C is a constant for a given polymer-solvent system.

- Cross-linkage density, v_e, that is the ratio between polymer density and M_c:

$$v_e = \frac{\rho_p}{M_c}$$

Hydrogel volumes are very variable because depending on the swelling capability of the system. The hydrophilicity of the network is related to the presence of chemical residues that can be found within the polymer backbone or on the lateral chains (Ganji, Vasheghani-Farahani, and Vasheghani-Farahani 2010), such as carboxylic (-COOH), hydroxylic (-OH), amidic (-$CONH_2$), sulphonic (-SO_3H) and others.

All those functional groups can affect the water retaining ability and the hydration process of a dry hydrogel network.

When a hydrogel in its initial dry, glassy state is in contact with solvent molecule, this latter attacks the hydrogel surface and penetrates into the polymeric network.

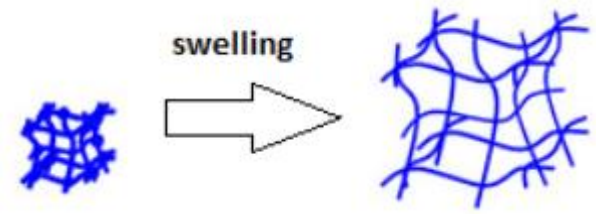

Figure 2.12: Swelling of a hydrogel network.

The meshes of the reticulum in the new formed rubbery phase start expanding, promoting the inlet of other water molecules. This additional swelling, however, is not a continuous process: against the favourable osmotic driving force, the volume increase is opposed by the presence of covalent or physical crosslinking, which like an elastic force, counteracts the stretching of the structure preventing its deformation. At equilibrium condition, the elastic forces balance the osmotic one, and no further swelling occurs.

This hydration process can be easily thermodynamically explained through the analysis of the chemical potential of the solvent (water). Its solvent spontaneous inlet inside the hydrogel is driven by a drop of its chemical potential, indeed the hydrated network has a lower value of Gibbs free energy than in the dry state. Meanwhile, chemical potential is increased by the elastic response of the polymeric chains, that opposes to the swelling. This phenomenon end when the chemical potential of water inside and outside the systems is equal (Patel and Mequanint 2011).

If the cross-linking degree increases, the swelling capability of gel decreases: the high density of bonds determines the enhancement of elastic force, so that the swelling equilibrium occurs at smaller amount of absorbed water.

Besides all those physicochemical characteristic, hydrogels must have some peculiar design criteria to allow control and reproduction of biological environment and a proper therapeutic compound delivery. Biocompatibility is a necessary feature, since they must not cause an immune host response with toxic or injurious effects. Along with this, hydrogel has to mimic well the extracellular matrix, to guarantee cell-compatibility and allow the mass transport of nutrient and gases into, out of and within the network, for the survival and proliferation of cells. Thus, matrix permeability is a very important design parameter, and it's strictly correlated to the mechanical properties and swelling behaviour of the reticulum. Also, biodegradability is a key-property for biomedical aim, because hydrogels have to be designed to degrade in physiological environment via ester hydrolysis, enzymatic hydrolysis or photolytic cleavage. This degradation provides the space to allow cell proliferation or controlled drug release of the loaded active compound (Kharkar, Kiick, and Kloxin 2013).

In general, hydrogels can be prepared from either synthetic or natural materials. Water-soluble linear polymers of both origins can be cross-linked to form hydrogels in diverse ways:

- Polymeric linking via chemical reaction;
- Using ionizing radiation to generate main-chain free radicals which are able to recombine as cross-linking junctions;
- Polymerizing monomer on the backbone of a preformed polymer, activated by the presence of chemical reagent of high energy radiation treatment;
- Through physical interaction, such as entanglement, electrostatic and crystallite formation, with or without heating.

Sometimes, synthetic hydrophobic monomers are used to regulate hydrogel properties for specific applications. Since they're stronger with respect to natural one, their degradation rate is slower, but on the other hand are more difficult to dissolve.

Hydrogels can be used for a great variety of applications in biomedical field thanks to their unique properties: biocompatibility, protection of active principle from hostile environment, controlled drug release in response to environmental physicochemical stimuli. An optimal design is required to obtain a hydrogel customized for the precise application.

3 Materials and methods

3.1 Polymeric nanoparticles synthesis

In this section, an overview of nanoparticles synthesis is presented, along with a brief description of polymers selected and their major features. Furthermore, also the stabilizing agents are issued, as ionic surfactant that confer electric charges to nanoparticles.

3.1.1 Poly (ethylene glycol) - PEG

It is a linear polymer obtained from the anionic polymerization of ethylene oxide and commercially available over a wide range of molecular weights. PEG is one of the most important polymers in the market also thanks to its wide-ranging possible applications.

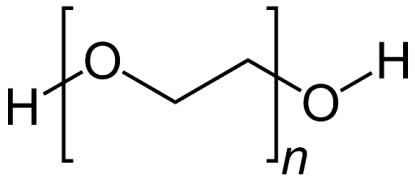

Figure 3.1: Poly (ethylene glycol).

PEG is soluble both in water and organic solvents, such as ethanol, benzene, dichloromethane.

It is generally considered biological inert and safe, although a minority of people are shown allergic response to the polymer itself or to side products formed during synthesis that lead to hypersensitivity. In any case, it has many advantages, such as good hydrophilicity, flexibility, antiphagocytosis against macrophages, resistance to immunological recognition and biocompatibility (Xiao et al. 2010).

There are many types of PEGs that are classified on the base of their average length, that is representative of the number or monomeric units present inside the macromolecule.

To this variation corresponds also the difference between some physical properties, as boiling point and viscosity.

For our purposes, the poly (ethylene glycol) methyl ether is used, that is a PEG with methyl functionalization. The molecular weight is 5000 Da.

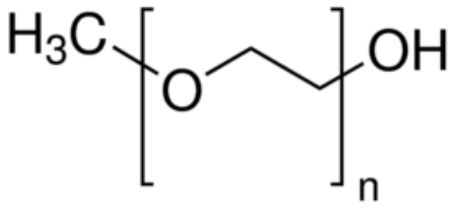

Figure 3.2: Poly (ethylene glycol) methyl ether.

3.1.2 Polylactic acid - PLA

Polylactic acid (PLA) is a biodegradable and bioactive thermoplastic aliphatic polyester, derived from natural and renewable sources, such as agricultural wastes.

It is produced from polymerization of the related monomer lactic acid, obtained generally from fermentation or chemical synthesis. Actually, fermentation is the preferred way to obtain such monomer because it can be synthetized in two configurations L(+) and D(-), that have different biological activity. In fact, only the L(+) conformation is biologically active and through fermentation the yield of this stereoisomer is much greater.

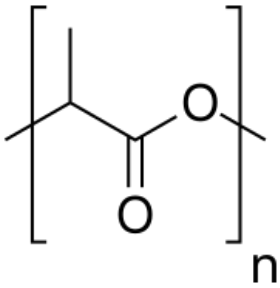

Figure 3.3: Polylactic acid.

Generally, PLA is obtained pure from polymerization of just L-lactic acid (Jamshidian et al. 2010) via condensation polymerization or ring-opening polymerization.

Due to its suitable biodegradability, low toxicity, low immunity and good mechanical strength, PLA has been approved by FDA for biomedical applications (Hyon 2000). On the other side, PLA presents low hydrophilicity and low drug loading for polar compounds, along with long degradation time.

3.1.3 PEG-PLA copolymer

For this work, PEG-PLA block copolymer is the chosen material for nanoparticle synthesis.

Through copolymerization with PEG, PLA can be improved in hydrophilicity and degradation rate. In addition, degradation products of PEG-PLA copolymer can enter the tricarboxylic acid cycle or can be eliminated by kidney. Thus, in low concentration, the copolymer is non-toxic and not accumulating *in vivo* (Ignatius and Claes 1996).

The copolymerization of PEG and PLA can increase the drug loading and prolonging residence time of active compound, avoiding them being engulfed by macrophages (Essa, Rabanel, and Hildgen 2011).

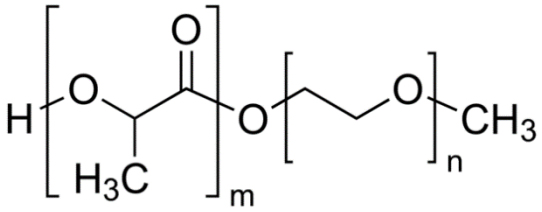

Figure 3.4: PEG-PLA copolymer.

3.1.3.1 PEG-PLA copolymer synthesis via ROP

The block copolymer PEG-b-PLA can be synthetized by ring-opening polymerization (ROP) of lactide monomer in presence of methoxy-terminated poly (ethylene glycol).

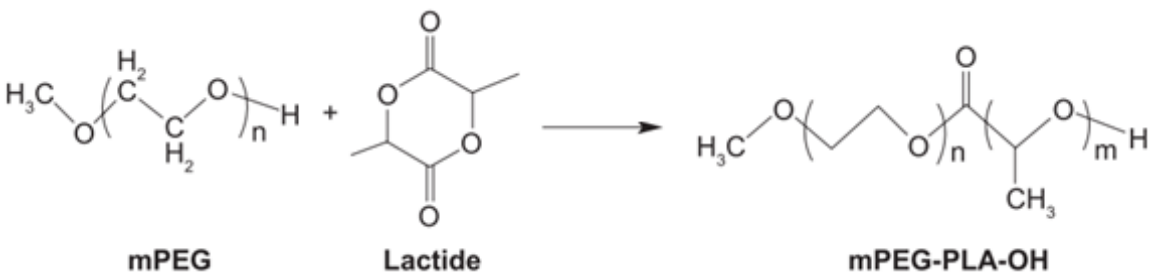

Figure 3.5: Synthesis scheme of PEG-b-PLA.

ROP is one of the most important paths to synthetize polymers. Those reactions are particular forms of chain growth polymerizations, although more complicated than those, very useful to obtain polymers with specific and controllable properties and to get synthetic variants of natural and biodegradable polymers. It is an effective way to obtain high molecular weight macromolecules, because one of the advantages is the absence of typical by-products of linear monomers polymerization (Nuyken and Pask 2013).

All ROP have in common that the monomers are cyclic molecules, no matter of their dimensions, although the driving force of the reaction varies according to the nature of monomer. Ring of 3-8 atoms may polymerize due to the loss of enthalpy associated with the loss of ring strain, in fact the ring monomer stability is one of the major driving force, so that for example cycloalkanes of three or four carbon atoms have a high reactivity, whereas a six-carbon atoms ring is a very intrinsically stable configuration.

In any case, a catalyst is needed to activate molecules and open rings, and according to the nature of this compound, it's possible to classify ROP as cationic, anionic or radical.

In this case the organo-catalyst is the 1,8-diazabicycloundec-7-ene, also known as DBU.

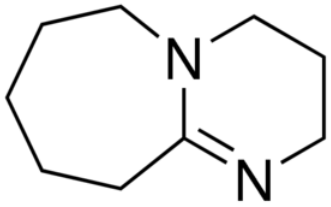

Figure 3.6: 1,8-diazabicycloundec-7-ene.

Experimental procedure starts with the dissolution of 0.5 g of poly(ethylene glycol) methyl ether (5000 Da) in 2 mL of dichloromethane (DCM), with the introduction of 20 µL of DBU. It occurs under magnetic stirring at room temperature.

At the same time, 1.666 mL of lactic acid (LA) are dissolved into 6 mL of DCM, under magnetic stirring, with mild heating (40-45 °C) and with the presence of a reflux condenser, due to the boiling point of the solvent, that is 40 °C.

Once solvation is over (it lasts about 10 min), the LA solution is added quickly to the PEG/DBU one and is allowed to stir rapidly for 10 min.

The reaction mixture is then quenched by addition of acetone (14 mL) and the PEG-b-PLA is recovered by precipitation from cold diethyl ether, collected by filtration and dried under vacuum to yield a white amorphous polymer.

DCM [mL]	PEG [mg]	LA [mL]	DBU [µL]	Acetone [mL]	Diethyl ether [mL]
8	500	1.666	20	14	*Quantum sufficit*

Table 3.1: Amount used in synthesis of PEG-b-PLA.

3.1.4 Stabilizing agents

Stabilizing agents are amphiphilic compounds able to reduce the surface tension of hydrophobic nanoparticles, improving their stability in water environment. An emulsifier (surfactant or tenside) contains both a water-soluble section (*head*) and a water-insoluble part (*tail*): they can diffuse toward the particle, inserting the hydrophobic tail inside the NPs core and exposing the polar head to water matrix, shielding the polymeric structure from the environment.

3.1.4.1 (2-hydroxyethyl) trimethylammonium chloride

(2-hydroxyethyl) trimethylammonium chloride, or choline chloride, is an organic compound and a quaternary ammonium salt that acts like a cationic surfactant.

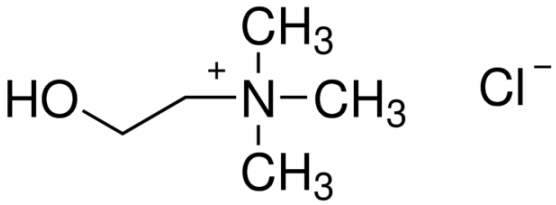

Figure 3.7: Choline chloride structural formula.

It is a salt that in water environment divide into choline and chloride ions, obtaining a polar head able to confer a net positive charge to the nanoparticles.

Its toxicological profile is almost null, since it's an important nutrient for human health as precursor of acetylcholine, thus approved by FDA for biomedical use.

3.1.4.2 Polysorbate 80

This compound, also known as TWEEN 80, is a non-ionic surfactant used both in biochemical and food application to disperse substances. It is extremely versatile and effective, while also being non-toxic, non-mutagenic, non-carcinogenic, with a very little potential for human skin irritation and sensitization.

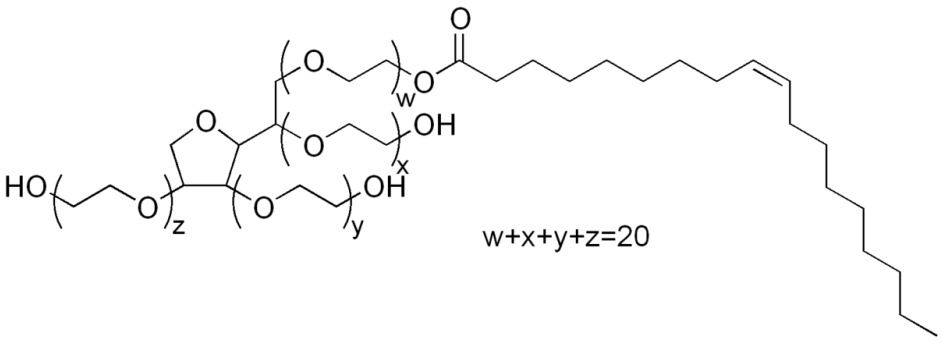

Figure 3.8: Tween 80 structural formula.

3.1.4.3 Sodium Dodecyl Sulphate - SDS

Sodium dodecyl sulphate is an anionic surfactant made by a sulphate group with a 12-carbon tail attached. It confers net negative charge to nanoparticles.

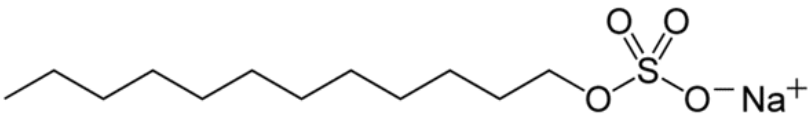

Figure 3.9: SDS structural formula.

SDS can irritate for prolonged usage or in hypersensitive individuals, but in daily life applications it is not considered carcinogenic.

Experimental studies point out that SDS at high concentration is cytotoxic: cells exposed to SDS shown, after a time, morphological changes, loss of membrane integrity, reduction of cell adhesion to substratum and reduction of mitotic cells.

3.1.4.4 *Cetyltrimethylammonium bromide - CTMAB*

As other emulsifier, this compound acts both as stabilizer but also as molecules that confers a net charge to the stabilized particle, in this case a positive charge.

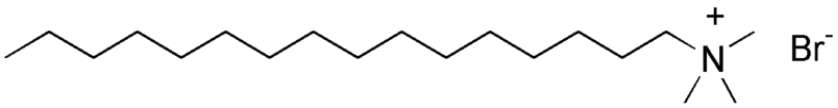

Figure 3.10: CTMAB structural formula.

As all other ammonium, quaternary salts can cause irritation and sensitization, but its toxicological profile has been considered safe for biomedical applications.

3.1.5 Polysaccharides

Natural occurring polymers exhibit some properties very desirable in tissue engineering and make them excellent candidates for biomedical applications. In fact, some of them are biodegradable, non-antigenic, non-toxic and also biofunctional, with hydrophilic properties and ample functional groups for further modifications (Q. Wang et al. 2011).

In this work, they have been used as surface modifiers, to produce opposite charge nanoparticles.

3.1.5.1 Sodium Alginate

It is an anionic linear polysaccharide typically obtained from brown seaweed and constituted by a multiblock copolymer of (1-4)-linked β-D-mannuronate (M) and C-5 epimer α-L-guluronate (G) residues.

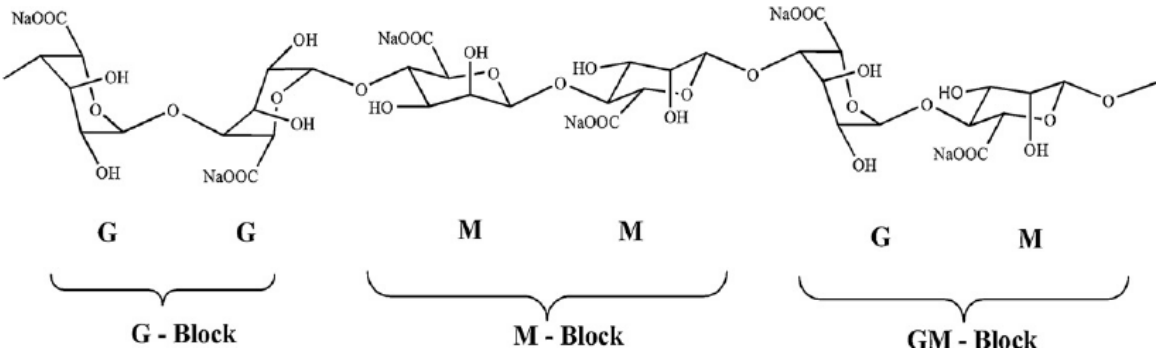

Figure 3.11: Sodium alginate structural formula.

Alginate presents carboxyl groups which may introduce negative charge to the polymer at appropriate pH values. It is a salt derivative effective to confer to nanoparticles a negative net charge. It is a very interesting molecule for biomedical applications because of its non-toxicity to humans (Lee and Mooney 2012).

3.1.5.2 Chitosan

It is a linear polysaccharide composed of randomly distributed β-(1→4)-glucosamine and *N*-acetyl-D-glucosamine. It can be obtained from chitin trough deacetylation, that have to reach at least 50%.

With respect to other presented polysaccharide, this macromolecule is soluble in water just with a slightly acidity (pH should be lower than 6) (Rinaudo 2006).

This is the only pseudonatural cationic polymer, because in acidic solution the amino groups become protonated and introduce positive charge to the polymer.

It has very interesting properties, such biodegradability and antibacterial activity, that make it a suitable material for biomedical applications.

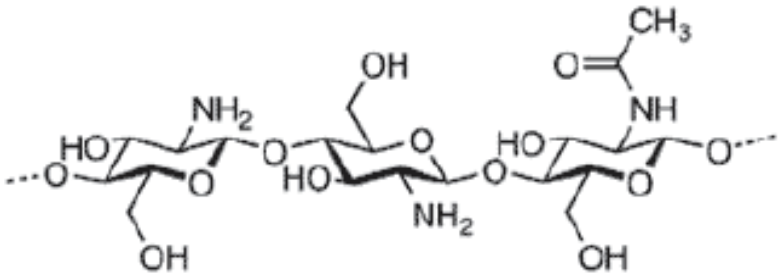

Figure 3.12: Chitosan structural formula.

3.1.6 Nanoparticle production

The aim of this work was to synthetize polymeric nanoparticle with a net surface charge through the physical absorption of tensides onto the NPs surface, which result also stabilised in aqueous medium.

Just two methods were used, starting always from the same polymer matrix to get a great variety of nanoparticles to be compared. No drug has been introduced, but in any case, a surfactant has been added to obtained charged particles.

Here, are listed all the synthesis pathways, divided according to the method for nanoparticle preparation descripted above.

3.1.6.1 Solvent diffusion method

Very simple method of synthesis, that can be easily adapted to different preformed polymers. From a detailed scientific literature screening, the final procedure is the following (McCall and Sirianni 2013).

It starts with the dissolution of 100 mg of PEG-PLA polymer in 1 mL of a suitable solvent, such as DCM or ethyl acetate. On the other side, 50 mL of an aqueous solution of surfactant is prepared and tenside amount can vary, according to the magnitude of the surface charge, from 0.3 to 0.6% w/v. In this case a great variety of emulsifiers can be exploited: in this work, TWEEN 80, SDS, CTMAB, choline and chitosan were used, according to the desired charge.

The obtained polymeric solution is then added dropwise to 1 mL of the surfactant solution, while vortexing the mixture to favour the homogenization of the new forming emulsion. After the entire mL of polymer solution has been added, continue vortexing the emulsion for 15 s.

Transfer quickly the emulsion to the sonicator to get a more disperse system using three series of 10 s each of sonication, in between which the probe should be cooled before proceeding.

Pour the polymer emulsion into the stirring emulsifier solution and left under hood to harden while stirring for three hours.

Nanoparticles can be collected through centrifugation for 15 min at 17.000 x g. Longer centrifugation times will result in the collection of higher fraction of smaller nanoparticles. Discarding the supernatant, nanoparticles are resuspended using deionized water and centrifuged again to remove the excess of surfactant and reducing progressively the total dispersant volume.

Another possibility to purified nanoparticles is to exploit the dialysis. Putting the polymer emulsion into a dialysis membrane of a proper cut off, it is possible to remove the excess of surfactant but without reducing the dispersant volume, that could be an advantage for the subsequent passage. In any case, it is proved in this work, that it is also possible to reduce the dispersant volume up to 25 mL without influence considerably the nanoparticle size.

A weight ratio of 1:2 threalose:polymer may be added at this point as a cryoprotectant before freezing and lyophilizing the system for storage.

3.1.6.2 Solvent evaporation method

Nanoparticle synthesis developed for the exploitation of biomacromolecule alginate and chitosan, it produces nanoparticles by precipitation into a polyelectrolyte solution, resulting in a coating of polysaccharides onto nanoparticle surface (Q. Wang et al. 2011).

Firstly, 100 mg of PEG-PLA is solved into 10 mL of acetone. Simultaneously, a 5 mL solution of alginate in deionized water is prepared. If chitosan is used instead of alginate, it should be dissolved in 0.2% w/v acetic acid solution. The concentration of different polysaccharides can be tuned according to the desired surface charge.

The polymer solution is then added to the aqueous one through a syringe pump, at constant rate under high stirring velocity.

The dispersion is left to stir under hood overnight to remove acetone through evaporation. The final system is simply made by nanoparticle coated with alginate or chitosan uniformly dispersed in aqueous medium.

Finally, nanoparticles can be collected through freeze-drying, obtaining a fine white powder.

3.2 Nanoclusters development

Nanoclusters are simply aggregates of polymeric nanoparticles, that occurs thanks to the difference of surface charge that has been provided during NPs synthesis.

These systems are obtained at high concentration of nanoparticles oppositely charged and stiffen in time through interparticle interactions, such as electrostatic forces and van der Waals attraction, which are the driving forces for the formation of nanoclusters.

The combination of those particles occurs almost randomly, so that the final system dimensions can be either in nanoscale or in microscale, although, generally, for biomedical applications nanoscale is preferred.

The synthesis is reported below (Q. Wang et al. 2011).

In two vials with 500 µL of deionized water each, positive and negative lyophilized nanoparticles, at reported concentrations, are dispersed at room temperature with magnetic stirring.

Then, the two dispersions are mixed in different proportions, to obtain different weight ratios to study. The system is left to stir for 30 min to homogenize nanoparticles and stored at 4°C for a day, to allow particles to be structurally organized before use.

Focusing on the application of nanoparticles with alginate and chitosan, three dispersions have been studied, designated as CA37, CA55 and CA73. The different mass ratio between positive and negative nanoparticles are reported into table 3.2.

	Chitosan	Alginate
CA37	30%	70%
CA55	50%	50%
CA73	70%	30%

Table 3.2: Different mass ratio of chitosan and alginate nanoparticles for nanocluster synthesis.

Different proportion have been challenged also for nanoparticles with surface absorbed tenside, in specific SDS and CTMAB, that having opposite charge with similar intensity, constitute suitable NPs for the synthesis of nanoparticles.

3.3 Experimental hydrogel formulation

After the preparation of required charged nanoparticles, it's possible to proceed to hydrogel synthesis. In this chapter, methods used to produce three dimensional networks are discussed. The final aim is to produce a composite system of hydrogel and charged nanoparticle, which are introduced into the hydrogel formulation and entrapped within the 3D network.

It has been validated from different studies that the combined used of both natural and synthetic polymers appears to be a good combination to enhance biocompatibility on one side and designing possibility on the other (Rossi, Perale, et al. 2011).

In this work, a combination of agarose and carbomer 974P is used to produce highly biocompatible and pH dependent hydrogel, specifically studied and characterized in literature for spinal cord injury repair applications (Rossi, Santoro, et al. 2011).

- Phosphate Buffer Saline (PBS): it's a water based salt solution containing sodium phosphate, sodium chloride, potassium phosphate and minor amounts of carbonates and other sodium salts. The buffer effect helps to keep pH constant in hydrogel synthesis; the osmolality and ions concentration of the solution usually match those of human body.

- Carbomer 974P: it is a cross-linked poly-acrylic acid containing carboxyl groups (65%) that make it a ionizable molecule. The molecular weight is 1 million Da.

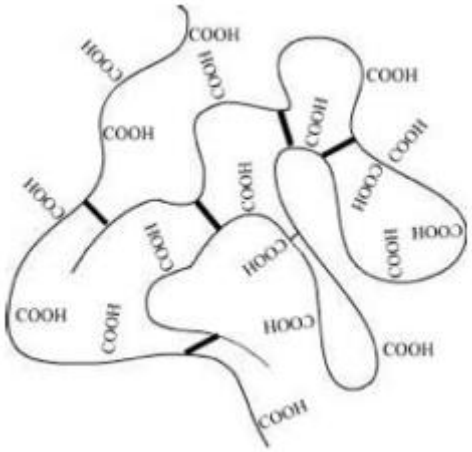

Figure 3.13: Carbomer structure.

- Polyethylene glycol (PEG): already descripted for the nanoparticle synthesis, in this preparation is used with a molecular weight equal to 2000 g/mol.
- Agarose: it is a purified linear galactan hydrocolloid isolated from agar or agar-bearing marine algae. Structurally, it is a linear polymer consisting of alternating D-galactose and 3.6-anhydro-L-galactose units.

  Agarose is used as gelling agent in biological applications, such as electrophoretic separation of nucleic acids and formation of gel plates for tissue cultures.

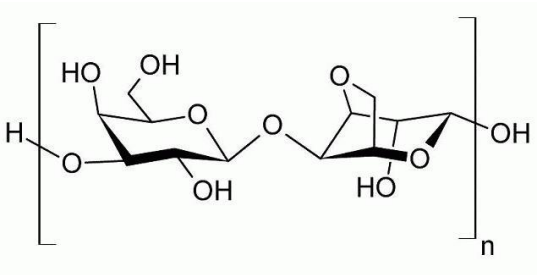

Figure 3.14: Agarose chemical structure.

- Carboxymethyl cellulose (low viscosity): it is natural polysaccharide containing carboxymethyl groups (-CH_2COONa) bound to some of the hydroxyl groups of the cellulose backbone.

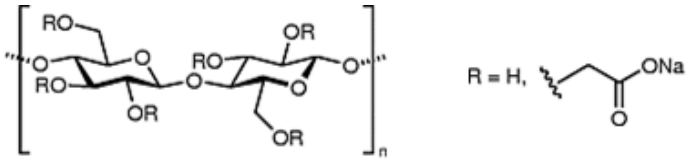

Figure 3.15: Carboxymethyl cellulose sodium salt chemical structure.

It is one of the water-soluble cellulose derivative that has a wide application in food and pharmacological fields due to its high chemical stability, non-toxic and hypoallergenic nature. It is safe, biocompatible and biodegradable with the additional value of being reproducible, abundant and cheap, coming from renewable sources.

In addition to those components, propylene glycol and glycerol are added as crosslinking agents.

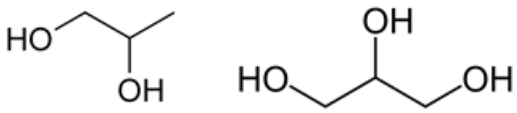

Figure 3.16: Propylene glycol and glycerol chemical structure.

Three different scaffolds are prepared, labelled as AC1, AC6 and AC6-CMC, according to the formulation and to the involved polymers for the constitution of final three-dimensional network. The procedures are illustrated below.

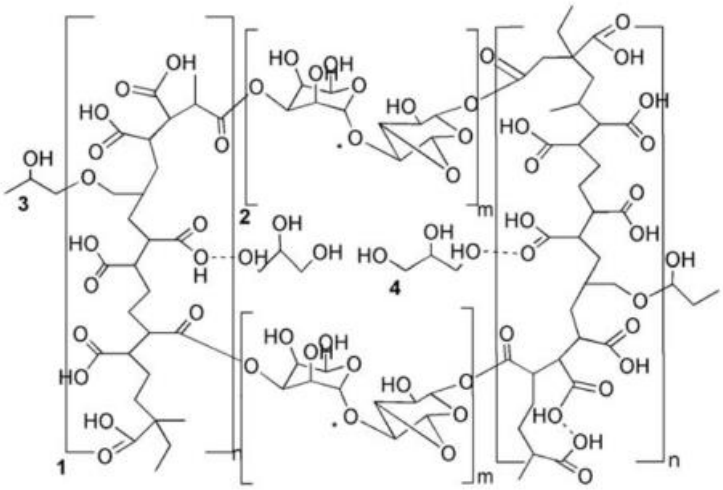

Figure 3.17: Scheme of 3D network formed via statistical polycondensation between carbomer 947P, agarose and cross-linking agents.

3.3.1 Hydrogel AC1

Used chemicals and their amount are shown in table 3.3:

PBS [mL]	Carbomer 974P [mg]	Propylene glycol [mL]	Glycerol [mL]	Agarose [mg]
9.95	50	0	0	20

Table 3.3: Amounts of chemicals used in hydrogel AC1.

The solvent for this formulation is PBS, Dulbecco's phosphate buffered saline solution, and only carbomer is involved to introduce the carboxyl groups needed to form cross-linking with hydroxyl groups of agarose, forming ester bonds and altogether giving rise to the three-dimensional matrix (chemical hydrogel).

Experimental procedure starts with the dissolution of 50 mg of Carbomer 974P into 9.95 mL of PBS. This occurs under stirring at high rates for 30 min, at room temperature. Once mixing is over, the solution is left to stabilize 1 h; after, pH is carried to 7.9 adding dropwise NaOH 1 N.

To 4 mL of this mixture, 20 mg of agarose powder are added and the mixture is then subjected to electromagnetic stimulation (microwave: 500 W irradiated power) for times between 30 seconds and 1 minute until boiling, heating up to 70-80°C. At this point gel is liquid and condensation reactions begin, through interconnections of hydroxyl groups.

The mixture is carried to 50°C and added with 4 mL of nanoparticle aqueous solution, in order to get a volumetric ratio of 50/50.

Amounts of the final solution equal to 500 µL are taken and placed in steel cylinders (as in figure 3.16), in order to confer a cylindrical shape to the hydrogels, and left at rest for about 45 min, until complete gelification.

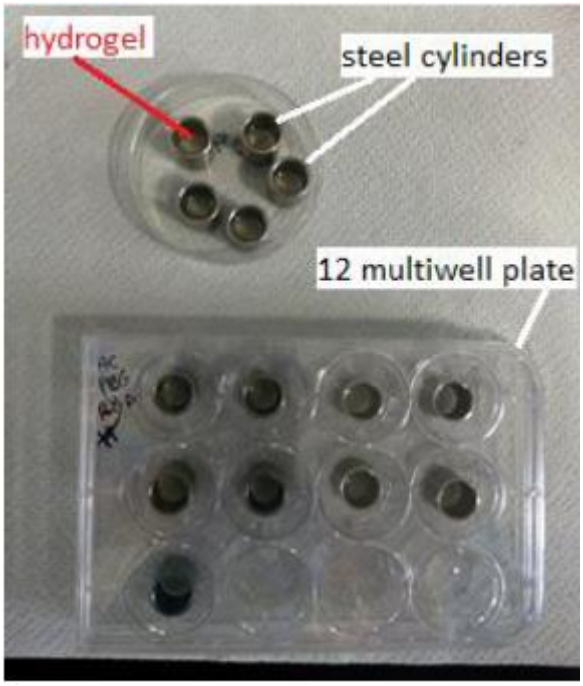

Figure 3.18: Hydrogel deposition in steel cylinders and an example of multiwell plate.

Hydrogels are finally removed from the cylinders and put into 12 multiwell plate (shown in figure 3.16), where 2.5 mL of distilled water are added to each well, to submerge hydrogel. The system is then left in rest in stove at 37°C with humid environment, to simulate biological condition to better evaluate nanoparticle release in time.

In this synthesis, the nanoparticle solution added has a NPs concentration of 40 mg/mL and for the same AC1 formulation, different kind of particles have been assessed, to compare their release behaviour with respect to the same hydrogel matrix. The same procedure is going to be applied for each hydrogel formulation.

3.3.2 Hydrogel AC6

This formulation sees the presence of two cross-linking agents in addition to the previous one. In table 3.4 are indicate their amounts:

PBS [mL]	Carbomer 974P [mg]	Propylene glycol [mL]	Glycerol [mL]	Agarose [mg]
6.85	50	3	0.1	20

Table 3.4: Amounts of chemicals used in hydrogel AC6.

As before, also in this case the three-dimensional network is promoted by esterification reaction that occurs between hydroxyl and carboxyl groups. With respect to the former formulation, now, hydroxyl groups are present not only in agarose, but also in propylene glycol and glycerol, so that the effect is to increase cross-linking possibilities, resulting in a more compact hydrogel structure. The influence the reacting system not only chemical, but also physical, increasing the viscosity. Indeed, before polymeric solution irradiation, polymer chains are not overlapped and segmental mobility is high. Increasing irradiation doses, intramolecular links and chain scissions are favoured. Thereby, the decrease of segmental mobility allows intermolecular crosslinks to be formed and give origin to local 3D matrix. Further irradiation increase privileged intermolecular crosslinking and chain scission, giving birth to macroscopic gels (Rossi, Perale, et al. 2011).

Therefore, the presence of cross-linking agents that increases viscosity, leads to a more difficult the segmental mobility of chains, promoting the formation of links between polymers and the obtainment of denser hydrogels.

In 6.85 mL of PBS solution, 50 mg of Carbomer 974P are dissolved under stirring at high rate for 30 min, until complete dissolution.

Then, 3 mL of propylene glycol and 0.1 mL of glycerol are added together to the mixture, which is kept under stirring for 30 min more and then left to settle for 1 h. NaOH is dripped inside to adjust pH value to 7.4.

Then, passages are the same as described in paragraph 3.2.1 for the production of hydrated hydrogel for nanoparticle release evaluation.

3.3.3 Hydrogel AC6+CMC

While the former hydrogel preparations were already well known in scientific literature, the introduction of carboxymethyl cellulose inside the synthesis is an innovation for biomedical applications. Many different formulations can be tested which differ in relative amount of component in order to define the best solution for the evaluation of nanoparticle release. Obviously, no characterization has already been done for those systems, thus the final results of this work can only give a qualitative idea of the final three-dimensional matrix mesh size and nature, by direct comparison with other release profiles.

Among all the tried formulations, the most promising one is obtained from the combination of the well-known AC6 hydrogel with the introduction of 25 mg of CMC with the usual 0.5% v/v of agarose. Greater amount of CMC results into a very unstable and labile structure, that tends to dissolve very rapidly.

The final amounts of compounds in the formulation are presented in the table 3.5:

PBS [mL]	Carbomer 974P [mg]	Propylene glycol [mL]	Glycerol [mL]	Agarose [mg]	CMC [mg]
6.85	50	3	0.1	20	25

Table 3.5: Amounts of chemicals used in hydrogel AC6-CMC.

The presence of CMC in this formulation acts like an additional source of carboxyl groups. Generally, increasing the amount of carboxyl groups in the formulation lead to higher values of swelling equilibrium, that is the matrix is not so dense as simple AC6 but result less compact, with the ability to retain more water (Rossi, Perale, et al. 2011).

All those considerations should be validated with specific analysis of morphological and rheological studies. In this work, just a simple comparison is done with respect to detailed characterized hydrogels.

The synthesis of this new formulation is right the same as AC6, with the only difference that in the first passage 50 mg of Carbomer 974P are dissolved in 6.85 mL of PBS with also 25 mg of CMC. This occurs under stirring for 30 min. Then, as before, 3 mL of propylene glycol and 0.1 mL of glycerol are added together, and the mixture is left to stir for 30 min. Finally, the solution is left at rest for 1 h, after which the pH is corrected to 7.4.

Hydrogel formation in the multiwell and its preparation of release analysis are not changed.

4 Results and discussion

The final aim of this work is to define the synthesis procedure and the features of polymeric hydrogel functionalized with biodegradable nanoparticles, in order to create a composite system for the controlled drug delivery.

This unconventional method for the drug administration is revealing to be a promising and versatile delivery platform for various active compound, that in this way are better controlled and the major possible therapeutic effect can be reached (Liu et al. 2007).

The two main actors are, as already described, nanoparticles and hydrogels, although present a well-known efficacy in nanomedicine field, singularly present some issues. The composite system created now is able to overcome all those drawbacks, producing a vehicle able to deliver both hydrophilic and/or hydrophobic molecules, in a specific site, at a defined rate that can be tuned and be different for dissimilar active compound, prolonging the final treatment of many diseases.

The characterization has been done primarily for nanoparticles, since the used hydrogel for the release evaluation have been already study in detail in many literature texts (Rossi, Santoro, et al. 2011). They're features are presented in any case, to better analyse the final release profile. The innovation presented in this work, as far as concerns hydrogel synthesis, is the formulation AC6+CMC, where carboxymethyl cellulose has been introduced to improve biocompatibility and biodegradability features.

4.1 PEG-PLA evaluation

From the H NMR analysis, it is inferred the successful synthesis of the PEG-PLA copolymer.

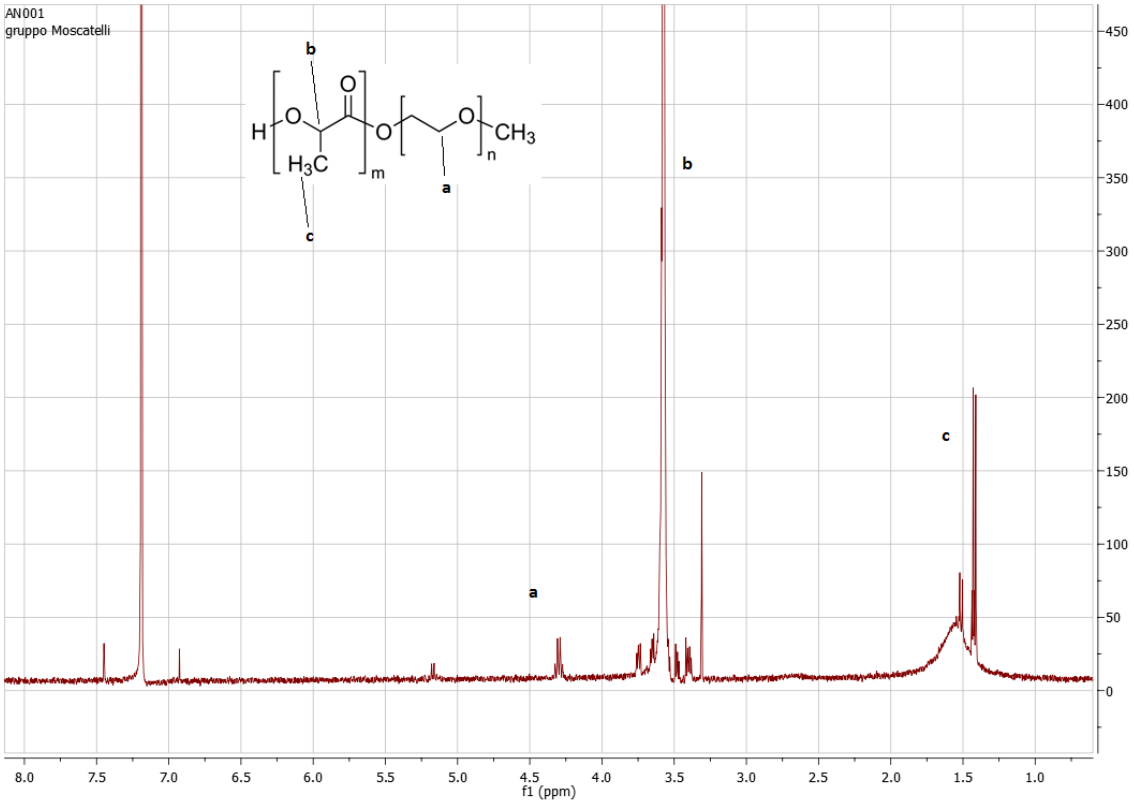

Figure 4.1: PEG-PLA NMR analysis.

Solvating a small amount of polymer in chloroform, H NMR is performed.

The peak at 5.2 ppm comes from one proton of each PLA monomer. The peak at 3.6 ppm represent the four protons of each ether PEG monomer. The three protons of the methyl group of each PLA monomer are responsible for the peak at 1.4 ppm.

4.2 Nanoparticles characterization

Physicochemical properties of nanomaterials contribute a lot towards they behaviour within biological milieu. Hence, adequate characterization of the nanoparticles is essential in order to obtain reliable data on formulation, cellular uptake, toxicity and dissolution.

Nanoparticles for drug delivery purposes should have some specific characteristics: some of them are biocompatibility, biodegradability and the protection of the active principle from the immune response of the organism.

All those features are provided from the formulation of the nanovehicle: in this case, the toxicological profile can be easily done looking to the components of nanoparticle. PEG-PLA has been recognised as a copolymer suitable for biomedical applications, and one of the great advantage of nanoparticles is to shield the active compound, screening its possible toxicity.

Indeed, during nanoparticle formation, the hydrophobic section of copolymer (PLA), exposed to water environment, coils up, creating a sort of polymeric sphere, whereas the hydrophilic part (PEG), highly hydrophilic, arranges all around PLA, stabilizing it, and increasing the hydrophilicity of the final nanoparticle. In this configuration, tensides molecule arranges themselves on the interface between the outer environment and the nanoparticle core, inserting in between PEG chains with the polar head outward.

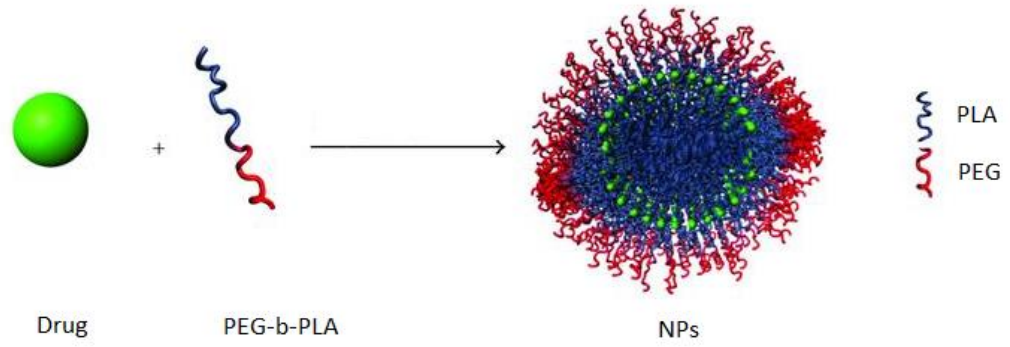

Figure 4.2: PEG-b-PLA drug-loaded nanoparticle conformation.

Another key factor is the particle dimension and the particle size distribution. This parameter is able to affect either the bioavailability and their distribution inside body, either the drug encapsulation efficiency (De Jong and Borm 2008).

According to the dimension of exploited polymer, nanoparticle size changes, and since the nanoparticle synthesis in not specifically governable, NPs dimension is not completely homogeneous and it is more appropriate to talk about a population of sizes with a certain polydispersity and mean value. Thus, for this reason, although nanoparticle size should be within 50 and 250 nm, as described in paragraph 1.1.3.1, this is just an average indication.

However, in this application, since nanoparticles are not free to move in the entire body but are confined from hydrogel scaffold in the injury site, also higher values of dimensions (350-400 nm) can be acceptable.

Along with those, since in this case it is important for final purposes, also the particle surface charge should be evaluated. More intense is the final charge, stronger interparticle/hydrogel interactions will be, but also more stable should be the system.

Conforming with the nanoparticle synthesis method, and above all on the surfactant used to confer a charge upon particle surface, both dimensions and charge can vary significantly, as demonstrated below.

4.2.1 Instrumentation

The main analysis methods used to characterize nanoparticles, is the Dynamic Light Scattering (DLS), also known as photon correlation spectroscopy or quasi-elastic scattering, and the Zeta Potential (ZP). These techniques have emerged as simple, non-invasive and executable under ordinary lab environments to investigate the hydrodynamic size and surface charge of NPs, respectively.

Both DLS and ZP measurements are based on the light scattering phenomenon, evaluating the Brownian motion of nanoparticles or macromolecules in solution and relates this motion to particles size and charge (Stetefeld, McKenna, and Patel 2016).

4.2.1.1 Dynamic Light Scattering - DLS

When a monochromatic beam of light encounters a solution containing a dispersed phase, NPs scatter incident light in all directions.

Indeed, when the light beam, that corresponds to an electromagnetic wave, hits the diffuser center (nanoparticles), the electrons inside the molecule move from the original position, creating a dipole.

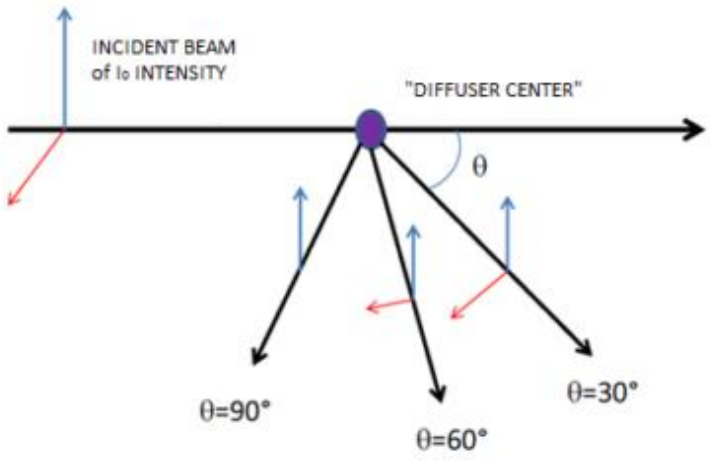

Figure 4.3: Diffusion of a light beam.

This dipole oscillates with the same frequency of the incident beam and radiates electromagnetic rays in all directions: the sum of all diffused beams is the scattered light.

Dimension and shape of particles influence the intensity and the scattered angle, since the intensity of scattered light is proportional to the 6th power of their radius.

Under the hypothesis that nanoparticles dimensions are much smaller than the wavelength if the incident light, Rayleigh's law can be applied to describe the relationship between the intensity of the diffused light and three main parameters: the scattering angle (angle between the incident light beam and the diffused light beam), the particles diameters and the incident light beam.

This assumption should be integrated with the hypothesis of unimodal distribution of particle sizes, because big particles can obscure the smaller ones.

$$\frac{i_\theta}{I_0} = \frac{8\pi^4}{\lambda^4 r^2}(1 + \cos^2\theta) * \left(\frac{n^2 - 1}{n^2 + 2}\right)^2 * \left(\frac{d}{2}\right)^2$$

Equation 4.1: Rayleigh's law.

Where: i_θ is the intensity of diffused radiation for the single "diffusion center", I_o is the intensity of the incident beam, θ is the scattering angle, n the refraction index of the particle, r the distance from the diffusion center, d the particle diameter and finally λ is the incident beam wavelength.

From this equation is possible to notice that the intensity of scattered light depends on the particle diameter and the incident beam wavelength.

In reality, particles in a colloidal dispersion are continuously moving and this can cause constructive or destructive interference, obtaining a fluctuation over time of scattered light intensity. These fluctuations are correlated against short decay intervals (τ), as visible in figure 4.3 (A).

In DSL instrument, a *correlator*, a digital component of the instrument, is used to derive the variations of diffused light in time and to transform them into a correlation function (Stetefeld, McKenna, and Patel 2016).

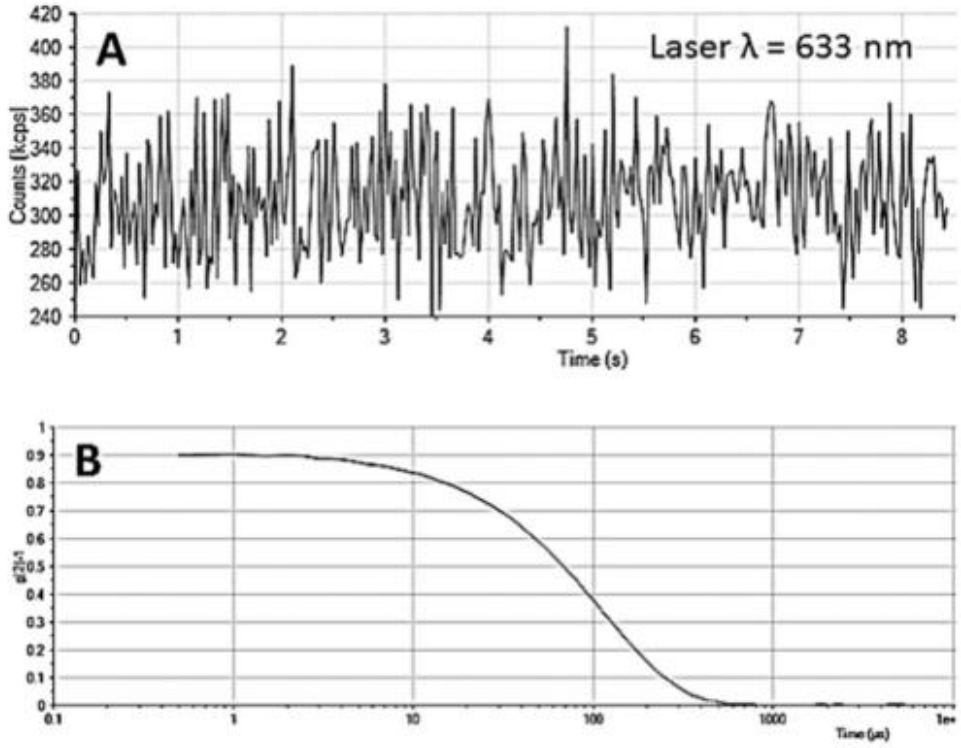

Figure 4.4: (A) Fluctuation of the scattered light by NPs due to consecutive destructive and constructive interferences. (B) The correlogram generated by the software in order to estimate the hydrodynamic radius.

The correlation function is the function that lies the particles' dynamics with the intensity of scattered light. If the analysis of signals is made in brief period of time, a good correlation, in the generated spectra, is visible (figure 4.3 (B)).

$$G1(\tau) = a[1 + b * \exp(-2D_t q^2 \tau)]$$

Equation 4.2: Correlation function.

Where a, b are constants depending on instrument and setting optics; q is the scattering vector, depending on the solvent refractive index, the light wavelength and on the scattering angle.

From the correlation function is possible to obtain Dt and, finally, applying the Stoke-Einstein equation, it's possible to identify the hydrodynamic radius R_H.

$$D_t = \frac{k_B T}{6\pi\eta R_H}$$

Equation 4.3: Stokes-Einstein equation.

From DLS, two main parameters are obtained: Z-average and polydispersity.

Z-average represent the average radius (or diameter) of the dispersed nanoparticles.

This parameter is not calculated as the average of the obtained diameters, but it is the average of the intensity of diffused light.

Polydispersity (PDI) represents the multiplicity, in terms of dimensions, between the particles contained in the sample.

$$PDI = \left(\frac{\sigma}{d}\right)^2$$

Equation 4.4: PDI definition.

PDI is defined as the ratio between the standard deviation (σ) of the population of nanoparticles from the average value of the diameter and the Z-average size.

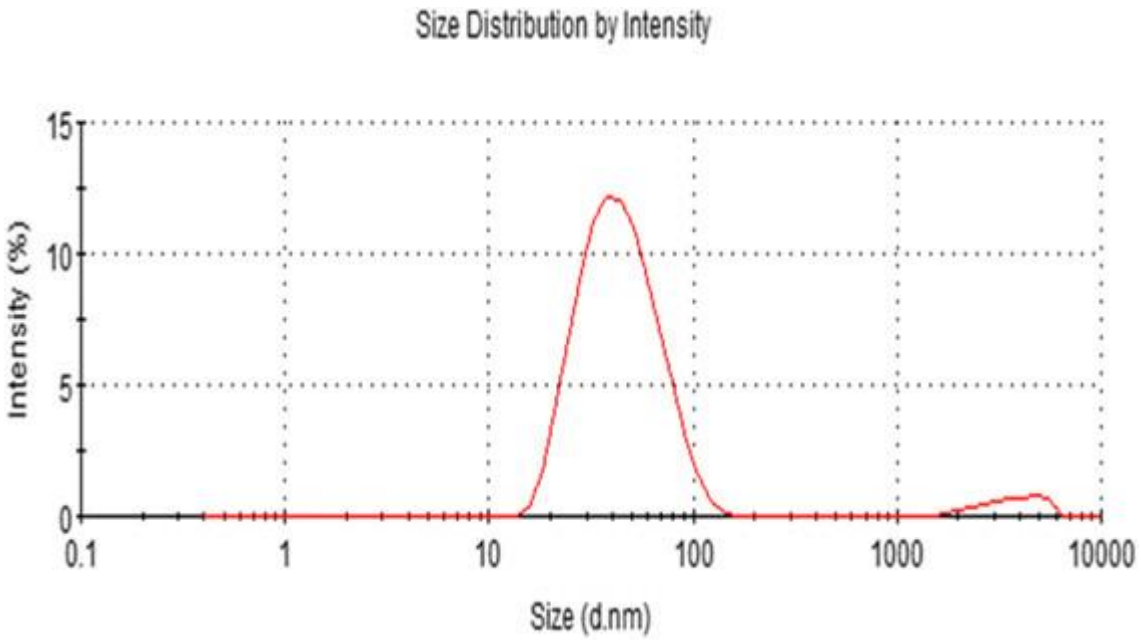

Figure 4.5: Nanoparticle population distribution.

4.2.1.2 Zeta potential – ZP

The development of a net charge at the particle surface affects at the distribution of ions in the surrounding interfacial region, resulting in an increased concentration of counter ions close to the surface. Thus, an electrical double layer exists around each particle.

The liquid layer surrounding the particle can be divided into two parts; an inner region, called Stern layer, where the ions are strongly bound, and an outer, diffuse, region where they are less firmly attached. Within the diffuse layer there is a notional boundary inside which the ions and particles form a stable entity. When a particle moves, ions within the boundary moves with it, but any ions beyond the boundary do not travel with the particle. This boundary is called the surface hydrodynamic shear, or slipping plane (Bhattacharjee 2016).

The potential that exists at this boundary is known as the Zeta potential.

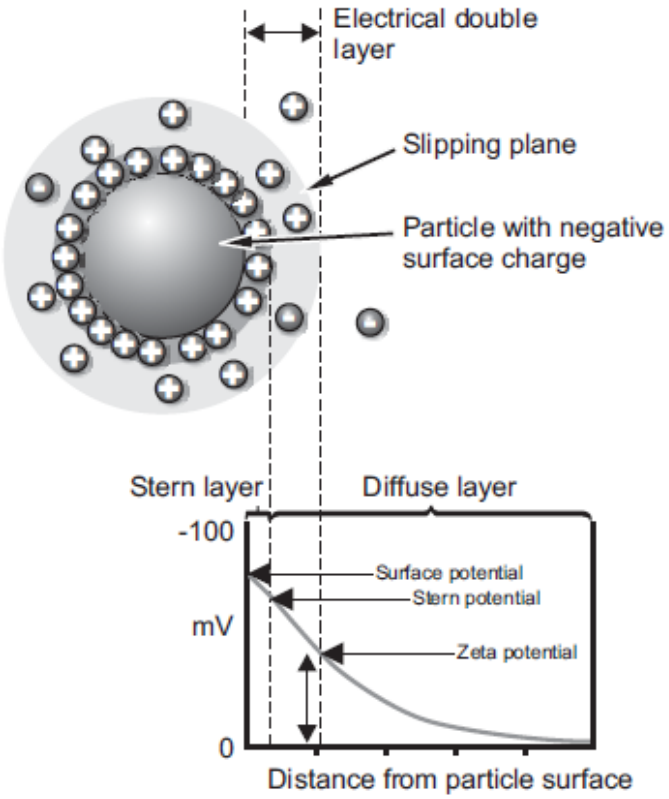

Figure 4.6: Electrical double layer representation with the slipping plane.

The magnitude of the ZP gives an indication of the potential stability of the colloidal system, and thus of the surface charge.

An important consequence of the existence of an electrical charge on particle surface is that they will exhibit certain effects under the influence of an applied electric field, that is also the exploited phenomena for the determination of ZP.

Indeed, when an electric field is applied across an electrolyte, charged particles suspended are attracted towards the electrode of opposite charge. Viscous forces acting against this movement, but when equilibrium is reached between these two opposite forces, the particle move with a constant velocity. This velocity is commonly referred to as Electrophoretic mobility.

From this knowledge, it is possible to obtain the ZP of the particle by the application of the Henry equation.

$$U_E = \frac{2\varepsilon z f(Ka)}{3\eta}$$

Equation 4.5: Henry equation.

Where z is the zeta potential, U_E is the particle velocity, ε is the dielectric constant of the medium and η its viscosity. Finally, $f(Ka)$ is the Henry's function, approximated at 1.5 for the Smoluchowski model approximation, good for dilute aqueous samples.

The essence of a classical micro-electrophoresis system is a cell with electrodes at either end to which a potential is applied. Particles moves towards the electrode of opposite charge.

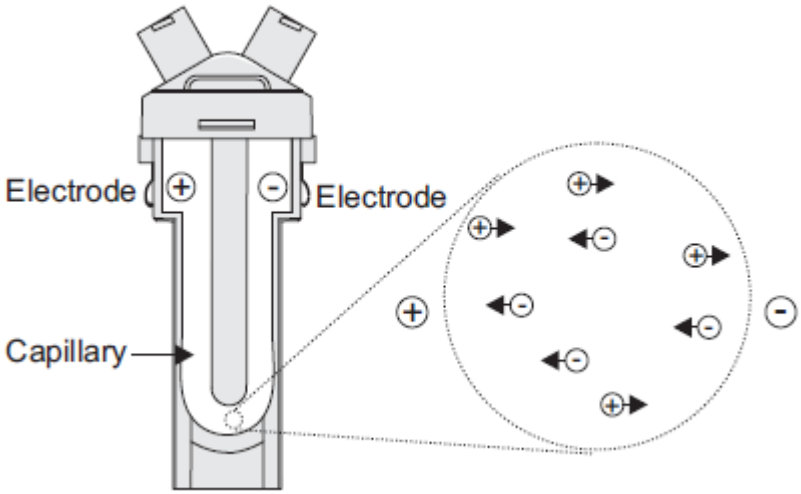

Figure 4.7: Electrophoretic mobility measurement principle.

The technique used to measure this velocity is the laser doppler velocimetry. The receiving optics is focused so as to relay the scattering of particles in the cell. The fluctuating intensity signal is detected, where the rate of fluctuation is proportional to the speed of particles.

Therefore, through those two techniques, it has been possible to characterize the nanoparticles used in this work.

To better relate the results, it has been necessary to develop a measurement protocol, to get comparable probes that differ just in the evaluated parameter. In addition, to get more precise values, the measurements are always tripled.

In this case, a simple standard plastic cuvette is washed with distilled water to avoid any possible contamination.

About 2.5 mg of lyophilized nanoparticle are inserted and solvated within 1 mL of distilled water. Then, it is inserted into the instrument where it is hit by the light beam.

The instrument temperature is set at 25°C and all the parameters needed to achieve the analysis are specified.

The instrumentation of DLS is presented in figure 4.7 below.

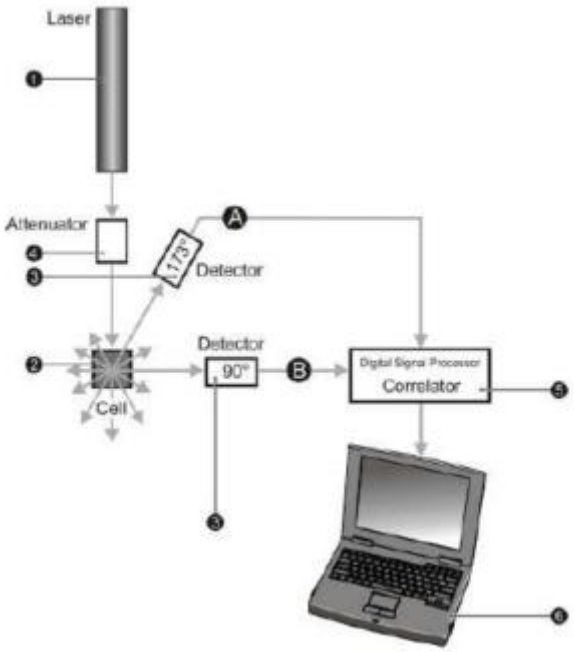

Figure 4.8: Dynamic Light Scattering

4.2.2 Results

Thanks to different methods of synthesis, the obtained nanoparticles are different in dimension and charge, so that it is possible to choose the better ones for subsequent application inside the hydrogels.

Splitting nanoparticle results according to different synthesis mechanisms, the majority of obtained nanoparticles comes from the solvent diffusion method. That's because it has been studied as the most promising one for its ability to obtain narrower particles distributions and also because of its versatility toward the use of various tenside.

PEG-PLA [mg/mL]	Tenside	Z-average [r.nm]	PDI	ZP [mV]	St. deviation [mV]
100	Tween 80 (0.3% w/v)	101.3	0.47	0.8	5.39

Table 4.1: Base synthesis results through solvent diffusion method.

It is possible to notice that the average size of those particles conforms perfectly to the final application, and since TWEEN 80 is an uncharged tenside, its action on the nanoparticle surface is just of thermodynamic stabilization, since no charge is present on the colloid.

Considering this starting synthesis of 100 mg/mL of PEG-PLA, with a concentration of tenside of 0.3% w/v, is the base for the subsequent modulations of those two parameters for the definition of the better nanoparticle formulation, evaluated through Z-average and ZP.

PEG-PLA [mg/mL]	Tenside	Z-average [r.nm]	PDI	ZP [mV]	St. deviation [mV]
100	Choline (0.3% w/v)	232.7	0.3	-4.97	3.75
200	Choline (0.3% w/v)	2382	1	52	6.57
100	Choline (0.6% w/v)	159.9	0.97	56.6	18.1
200	Choline (0.6% w/v)	290.8	0.65	3.65	4.48

Table 4.2: Nanoparticle synthesis through solvent diffusion method with choline in different concentrations.

It is evident how the presence of a cationic surfactant can change the final properties of nanoparticles. Just substituting choline to TWEEN 80, both dimensions and charge change, although for this final parameter, there is no and substantial difference from the base synthesis. This behaviour can be seen also doubling the concentrations of both polymer and emulsifier, maybe due to an ineffective absorption of tenside onto the particle surface.

An increased size on nanoparticles, that exceeds also in microscale, is obtained doubling the concentration of polymer: the presence of 0.3% w/v of choline is not enough to promote and stabilize smaller nanoparticles, that for this reason create larger systems.

The best options in this case is achieved using the same concentration of copolymer with respect to the base case, but doubling the concentration of tenside. In this way, not only the average radius is slightly decreasing, conforming better to the final aim, but also the surface charge is enlarged up to a considerable value, that allows the establishment of electrostatic forces.

Passing to anionic surfactant, SDS has been exploited.

PEG-PLA [mg/mL]	Tenside	Z-average [r.nm]	PDI	ZP [mV]	St. deviation [mV]
100	SDS (0.3% w/v)	201.8	0.42	-12.8	6.45
100	SDS (0.6% w/v)	160.4	0.47	-27.7	5.69
200	SDS (0.6% w/v)	70.9	0.19	-21.5	6.45

Table 4.3: Nanoparticle synthesis through solvent diffusion method with SDS in different concentrations.

From those synthesis, it is restated that an increase of surfactant concentration leads to smaller nanoparticles with a raised surface charge. Doubling the concentration of polymer, the final results present an important difference in size from the former probe, although it is also evident that to achieve a significant surface charge to allow both colloidal stabilisation and electrostatic potential a higher concentration of tenside is needed. However, each of those nanoparticles has the proper size to be applied in the hydrogel composite system, so the final evaluation must be done on the whole pool of nanoparticles.

Lastly, the CTMAB has been used as alternative cationic surfactant to choline, whose results are quite questionable. Thus, to have a counter evidence of the effect of cationic emulsifier on nanoparticles characteristics, also this compound has been challenged.

PEG-PLA [mg/mL]	Tenside	Z-average [r.nm]	PDI	ZP [mV]	St. deviation [mV]
100	CTMAB (0.3% w/v)	844.6	0.96	36.9	12
100	CTMAB (0.6% w/v)	273.4	0.45	28.3	6.27

Table 4.4: Nanoparticle synthesis through solvent diffusion method with CTMAB in different concentrations.

From the base synthesis with the introduction of this new tenside, formed nanoparticles are just too large to find application on the biomedical field, and for the internalization in hydrogel, although the presence of swelling behaviour, the expected release of those particles should be null. For this reason, following the trend of the decreasing dimensions as emulsifier concentration increases, doubling the CTMAB the result is a suitable radius, comparable to other nanoparticles' ones, that permit a direct comparison once inserted inside a hydrogel matrix.

To exploit also polysaccharide functionalization, this synthesis method has been tried similarly with chitosan, but the final dimension is exceeding the microscale (2895 r.nm), although the surface charge is suitable for final purpose (49.3 mV).

Therefore, the absorption of alginate and chitosan into nanoparticle surface has been proved via solvent evaporation method.

PEG-PLA [mg/mL]	Tenside	Z-average [r.nm]	PDI	ZP [mV]	St. deviation [mV]
10	Alginate (0.2% w/v)	175.4	0.4	-54.9	10.6
10	Chitosan (0.2% w/v)	641.4	0.54	37	2.61

Table 4.5: Nanoparticles synthesis through solvent evaporation method with alginate and chitosan.

With this method, it is possible to obtain chitosan coated nanoparticles with more useful dimension for biomedical application, although in any case the chitosan high molecular weight is preventing the production of smaller particles. On contrary, using alginate it is possible to get radii comparable to that obtained with solvent diffusion method.

On the other hand, the zeta potential is revealing that the surface charge is good for the subsequent exploitation of electrostatic forces for the formation of nanocluster and for the internalization in hydrogels.

From a comparison between all the different nanoparticles, just four of them have been selected for the subsequent application in a composite system. To get an idea of the release behaviour of different charged nanoparticles from anionic hydrogel, two of them coated with tenside are used and two with polysaccharide physic functionalization. Obviously, both alginate and chitosan nanoparticles from solvent evaporation methods have been chosen, on the other side from solvent diffusion, the best choice are that with smaller dimensions and with surface charge of at least 20 mV, to get enough driving force to promote hydrogel interaction.

For this reason, chosen particles are listed in table 4.6 below and their size distribution are shown in figure 4.8; 4.9; 4.10; 4.11 respectively.

	PEG-PLA [mg/mL]	Tenside
NPs-SDS	200	SDS (0.6% w/v)
NPs-CTMAB	100	CTMAB (0.6% w/v)
NPs-Alginate	100	Alginate (0.2% w/v)
NPs-Chitosan	100	Chitosan (0.2% w/v)

Table 4.6: Nanoparticles for hydrogel release evaluation.

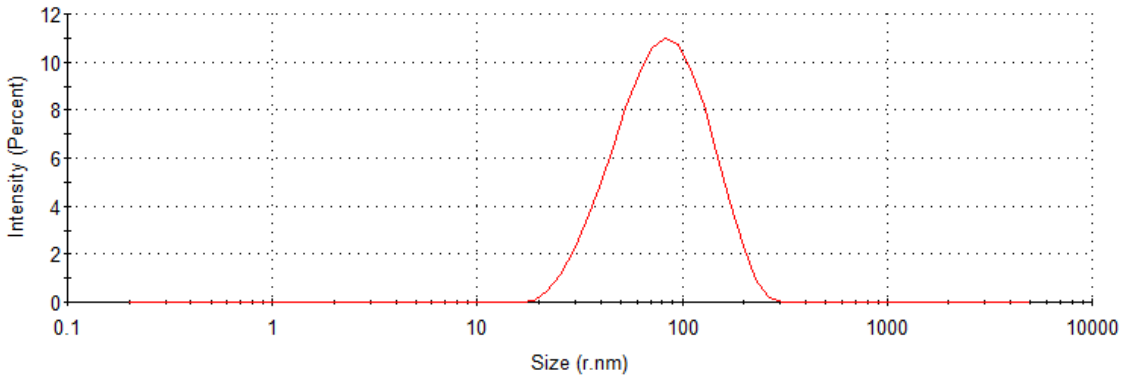

Figure 4.9: Size distribution by intensity of NPs-SDS.

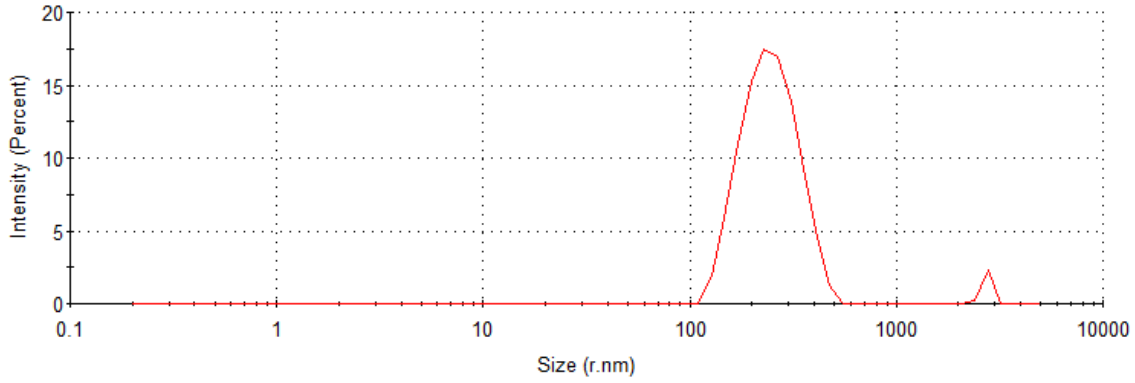

Figure 4.11: Size distribution by intensity of NPs-CTMAB.

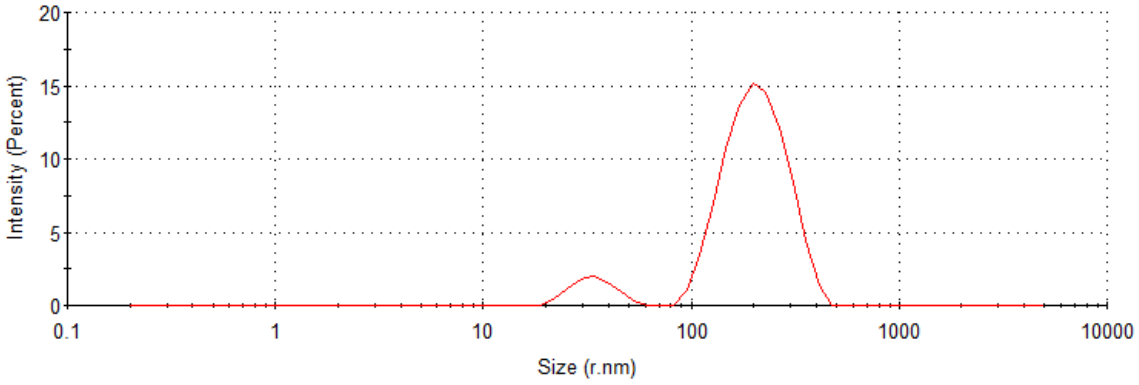

Figure 4.10: Size distribution by intensity of NPs-alginate.

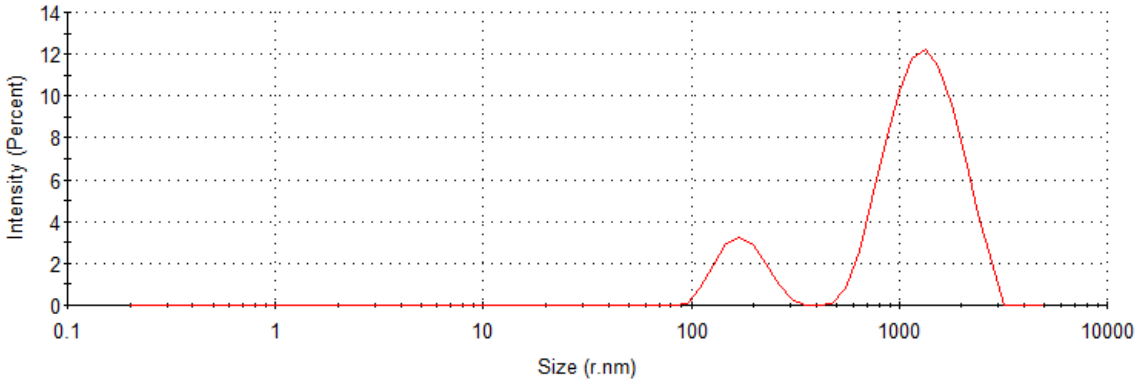

Figure 4.12: Size distribution by intensity of NPs-chitosan.

4.3 Hydrogel release behaviour

Once the nanoparticles have been synthetized and characterized, their release from gel with distinctive features is evaluated via the direct measurement of their concentration in the hydrogel well in time.

Indeed, as reported in paragraph 3.3, hydrogels, as soon as prepared, are inserted in wells and covered with a reference volume of distilled water, needed to have comparable withdrawal from each hydrogel.

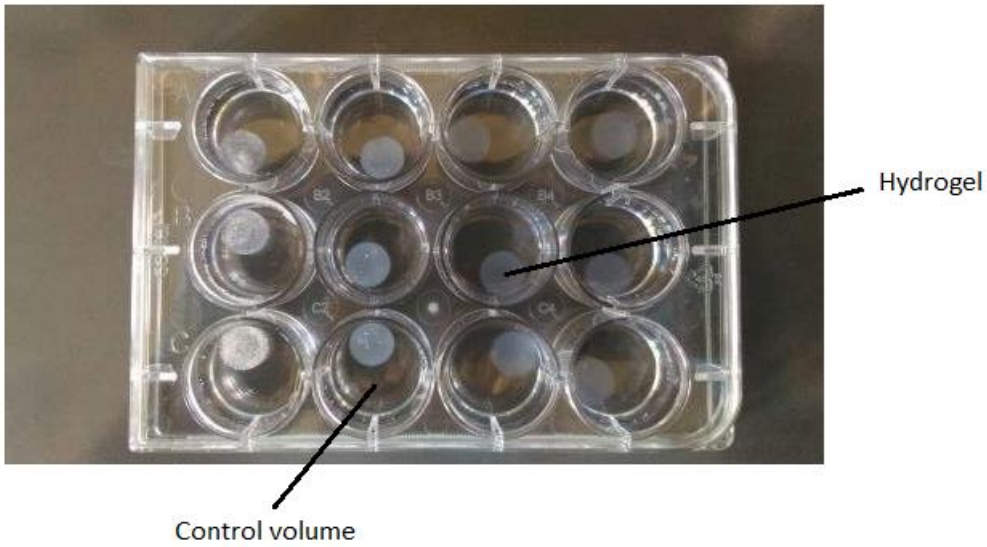

Figure 4.13: Multiwell containing hydrogels.

It has been found that a direct correlation exists between the nanoparticle concentration inside a sample and the intensity of scattered light detected in DLS. Thus, a simple way to analyse those probes is to exploit, indirectly, the measurements of the Dynamic Light Scattering and then apply a very plain equation to estimate the concentration.

To do this kind of assessment, it is necessary to identify a calibration curve that works as reference for the interpretation of conclusive results. This has been done for each of selected nanoparticles.

NPs have been diluted in 1 mL of distilled water in different concentrations: from 10 mg/mL to 0.0781 mg/mL halving each time the amount of nanoparticles. In addition, for each NPs, attenuator and position setting of light beam for the analysis of probes must be fixed, to ensure the linear dependence.

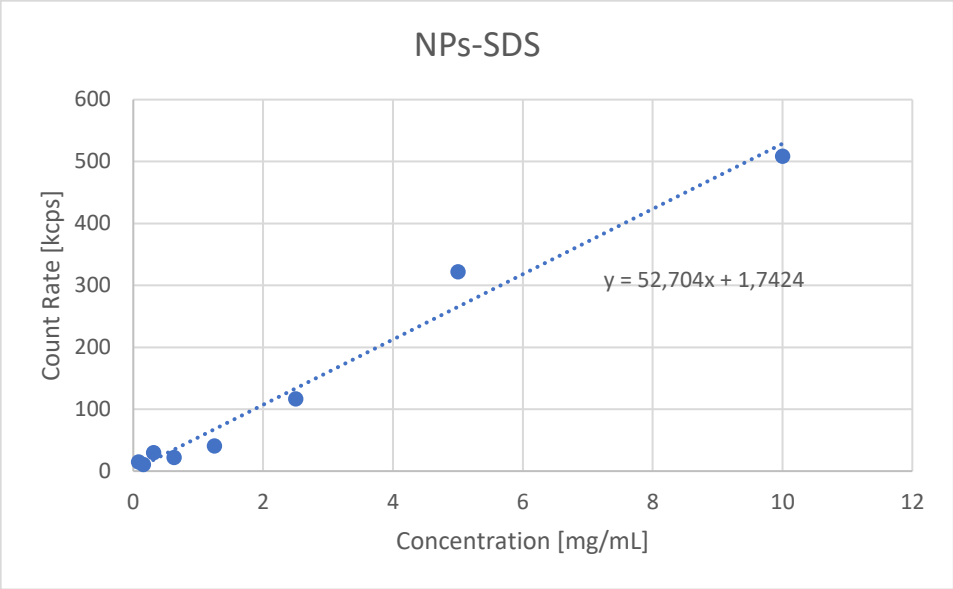

Figure 4.14: Calibration curve for NPs-SDS.

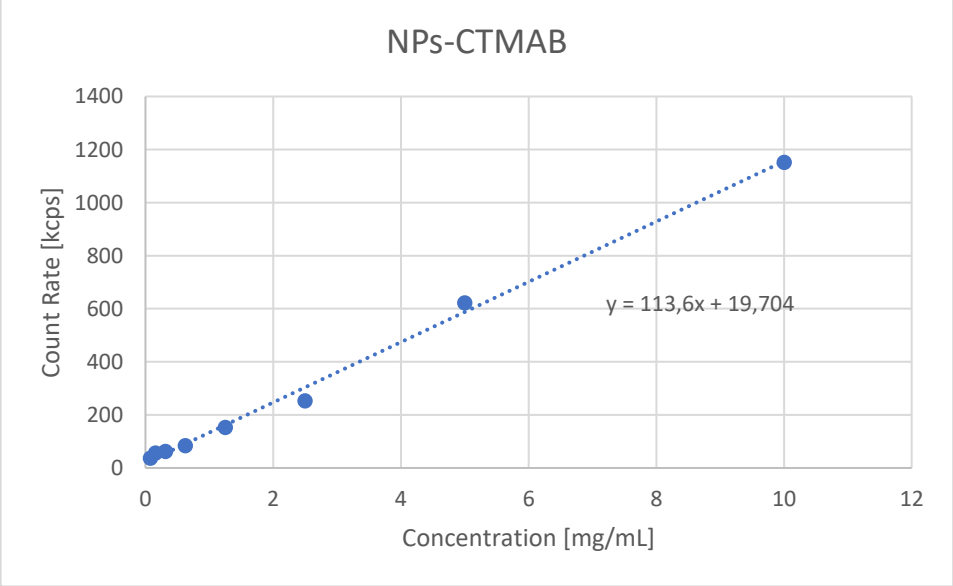

Figure 4.15: Calibration curve for NPs-CTMAB

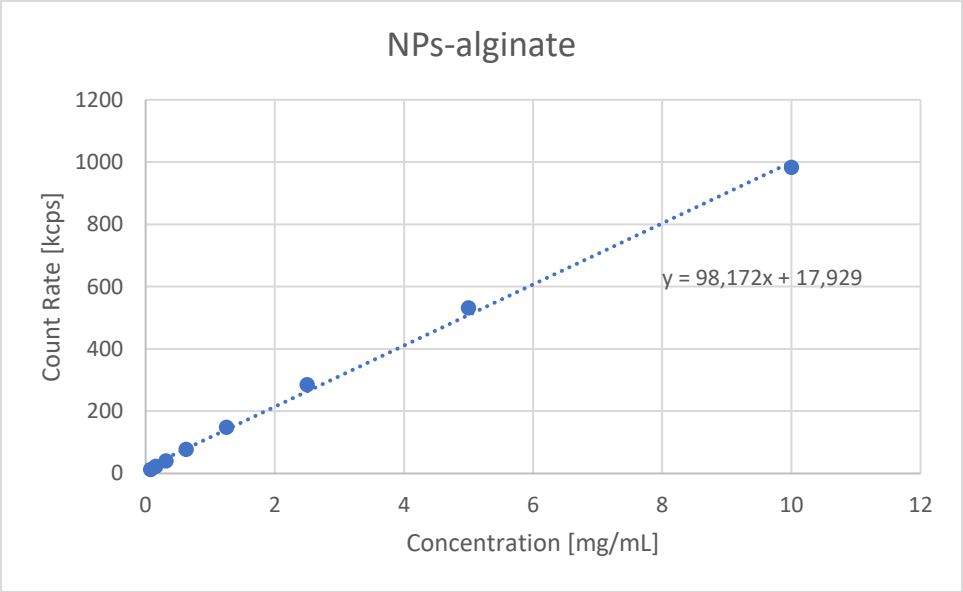

Figure 4.16: Calibration curve for NPs-alginate.

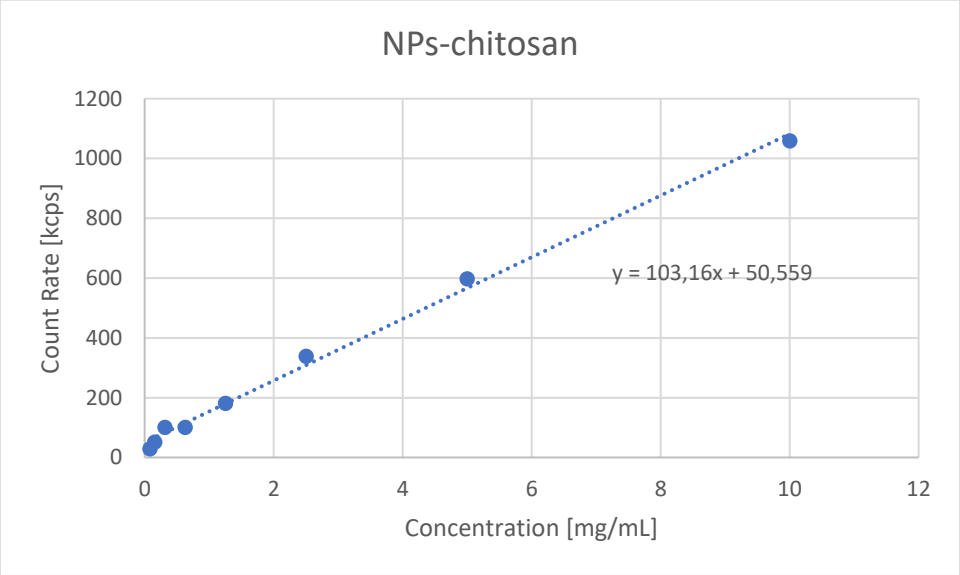

Figure 4.17: Calibration curve for NPs-chitosan.

For all of those calibration curve, the line equation is highlighted, because its parameters (intercept and slope) are characteristic for each nanoparticle:

$$count\ rate = m * concentration + q$$

Therefore, the procedure is based on the DSL analysis of 1 mL of NPs solution withdrawal from which the count rate is achieved. From this latter, through the calibration curves, it is possible to infer the concentration of NPs within that probe.

This operation is repeated in defined moments to get enough data to build a release profile curve, as concentration in function of time.

4.3.1 Release profiles from AC1

AC hydrogels, generally, are formulated in PBS solution and thus, the condensation reaction occurs in this electrolytic medium, developing esterification bond and hydrogen bonding between the polymeric chains. PBS solution salts, freely solvated in water, cause salt carboxylates formation and due to these reactions, AC hydrogels result quite anionic. This electrostatic behaviour influences the ability and the kinetic involved in nanoparticles delivery (Rossi, Santoro, et al. 2011).

AC1 hydrogel has been synthetized without the use of any cross-linker and, thus, the formulation is just involving carbomer 974P and agarose. This constitutes the main difference with respect to other presented hydrogels, and can be briefly described through the ratio between hydroxyl and carboxyl groups (A/B). This parameter affects not only the microchemistry but also the physical properties, and above all the ability to absorb and retain water along with the swelling behaviour (Rossi et al. 2013).

AC1 presents a low A/B ratio, thus the swelling equilibrium is quite great, reaching also the 4500% of the initial dry volume. This phenomenon is due to the less cross-linking density, that promotes the absorption of a greater amount of water before the recalling forces of linking point stop the expansion. Lower is the cross-linking density, greater is the mesh size and also the average molecular weight between two consecutive cross-linking points. Therefore, the time needed to reach swelling equilibrium is also increased.

For the aim of this work, the AC1 present a mean mesh size of 44 nm.

The already characterized selected nanoparticles have been loaded into AC1 formulation and physically internalized within the hydrogel matrix. Both electrostatic interaction and steric hindrance play a role in final release behaviours, as shown below.

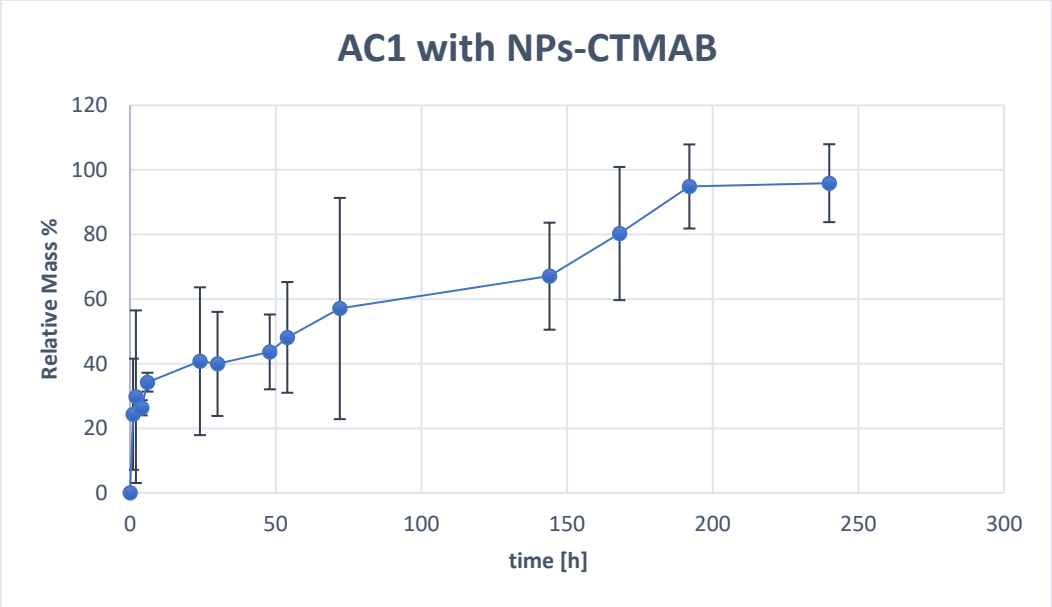

Figure 4.18: Release profile of NPs-CTMAB from AC1.

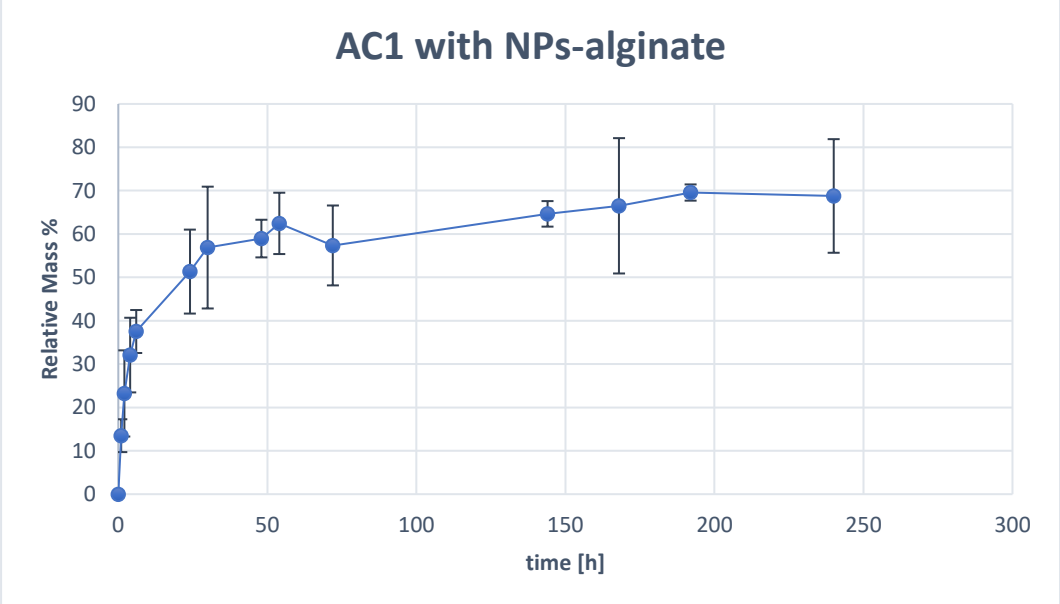

Figure 4.19: Release profile of NPs-alginate from AC1.

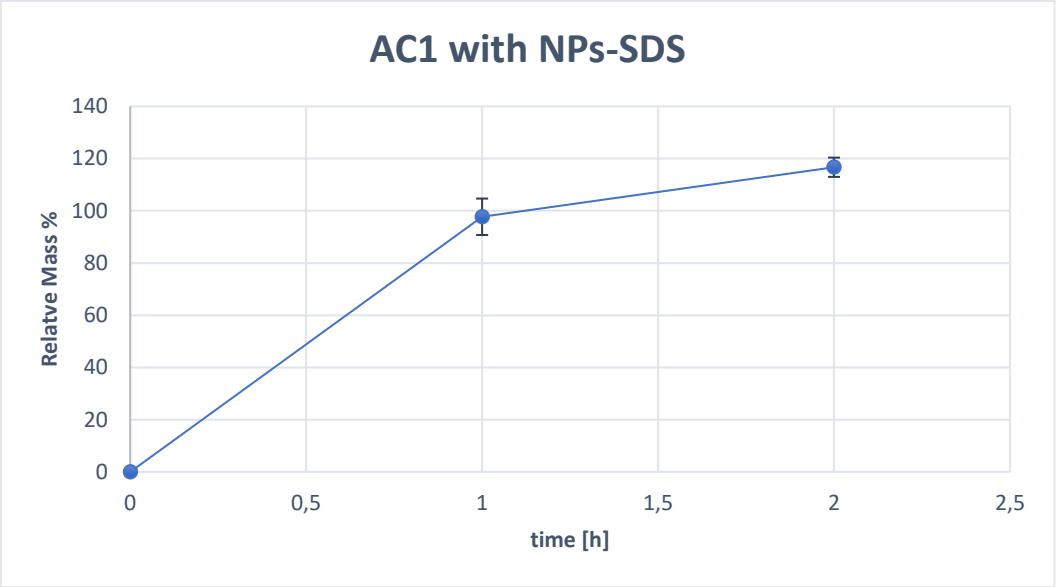

Figure 4.20: Release profile of NPs-SDS from AC1.

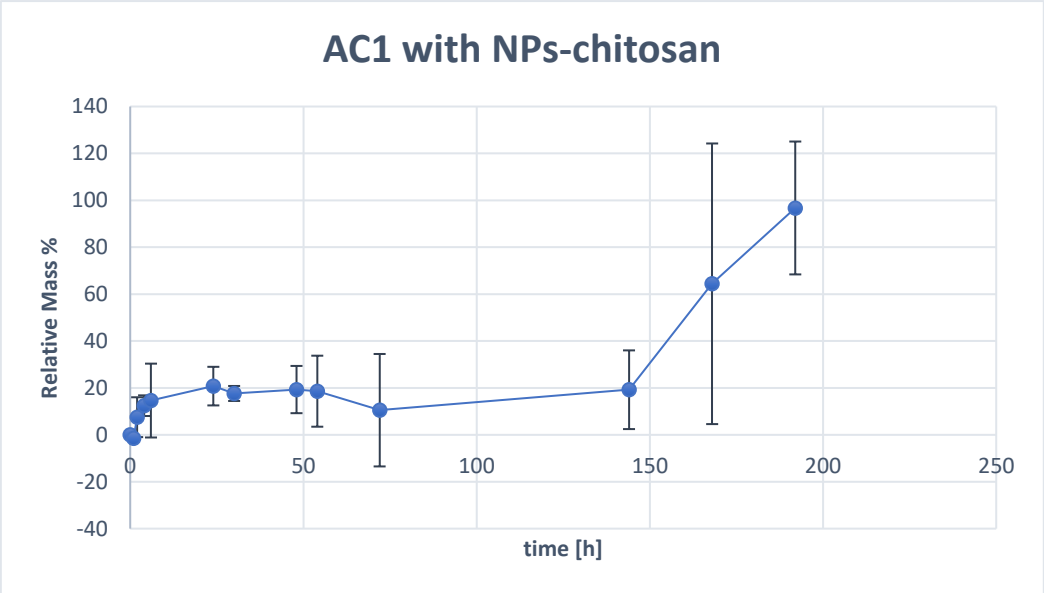

Figure 4.21: Release profile of NPs-chitosan from AC1.

From figure 4.19, the typical release profile can be seen, with a rapid initial release due to the initial high concentration gradient, leading to a burst effect.

It can be caused by nanoparticles that are at or near the solvent-hydrogel interface and thus could rapidly escape into the supernatant solution, or nanoparticles that have been able to find a fast path through larger pores of hydrogel, with respect to others constrained in smaller one. This burst effect is promoted also by the repulsive electrostatic interaction between hydrogel matrix, strongly anionic, and the charged nanoparticles (-54.9 mV).

After this initial huge outbreak, the release becomes slower and reaches an almost steady state condition at about 30 h from the synthesis (*plateau*). Moreover, it can be notice that almost all loaded nanoparticle has been released in 240 h, with except of that probably within the core of hydrogel. This confirms indirectly the absence of chemical stable interaction between nanoparticles and hydrogel matrix.

This evidence is visible also in the very fast release of NPs-SDS in figure 4.20, where the complete deliver of all the nanoparticle payload occurs in just 2 h from the synthesis. Also in this case, the electrostatic repulsion plays a fundamental role, but the faster release with respect to NPs-alginate now is due not to electrostatic interaction, that should promote a weaker repulsion for SDS (-21.5 mV), but to the dimensions, that for NPs-SDS are much smaller.

Moving away from this typical trend, both NPs-CTMAB (figure 4.18) and NPs-chitosan (figure 4.21), presents a step behaviour with the usual initial burst release of lower intensity with respect to the former profiles, followed by a plateau that is representing a sort of pause in nanoparticle release. This is due to both electrostatic interactions, that now promote nanoparticles to stay within the hydrogel matrix, and this can explain the lesser intensity of initial release. On the other side, also the steric hindrance of NPs is now very important, above all for chitosan coated ones: the release of large sized particles can occur just after the disintegration of hydrogel network, since the mesh size is too small to allow the passage of such big nanoparticles.

4.3.2 Release profiles from AC6

AC6 hydrogel presents a performance completely opposite with respect to AC1. Although the anionic electrostatic behaviour is equal, the presence of cross-linking agents in the formulation is increasing the ratio between the hydroxyl and carboxyl groups (A/B), leading to a denser cross-linked matrix and thus to a much smaller average molecular weight and mesh size, that now is of 9 nm. The swelling equilibrium is reached much faster than AC1 and also the amount of water absorbed is lesser.

Therefore, it is predictable that the release of big nanoparticles has a slower kinetic, although the same influence can be expected from the electrostatic interactions.

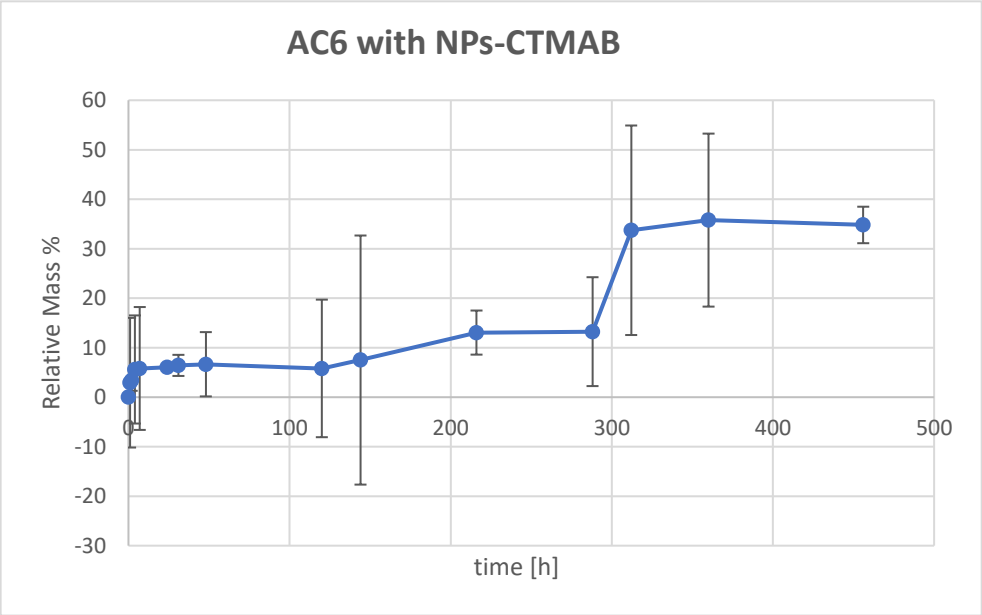

Figure 4.22: Release profile of NPs-CTMAB from AC6.

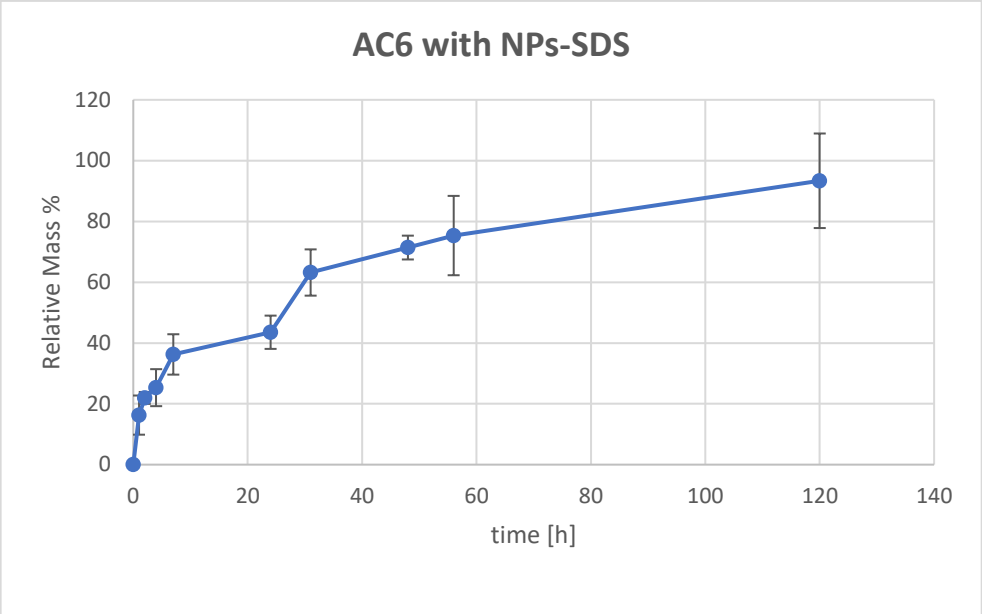

Figure 4.23: Release profile of NPs-SDS from AC6.

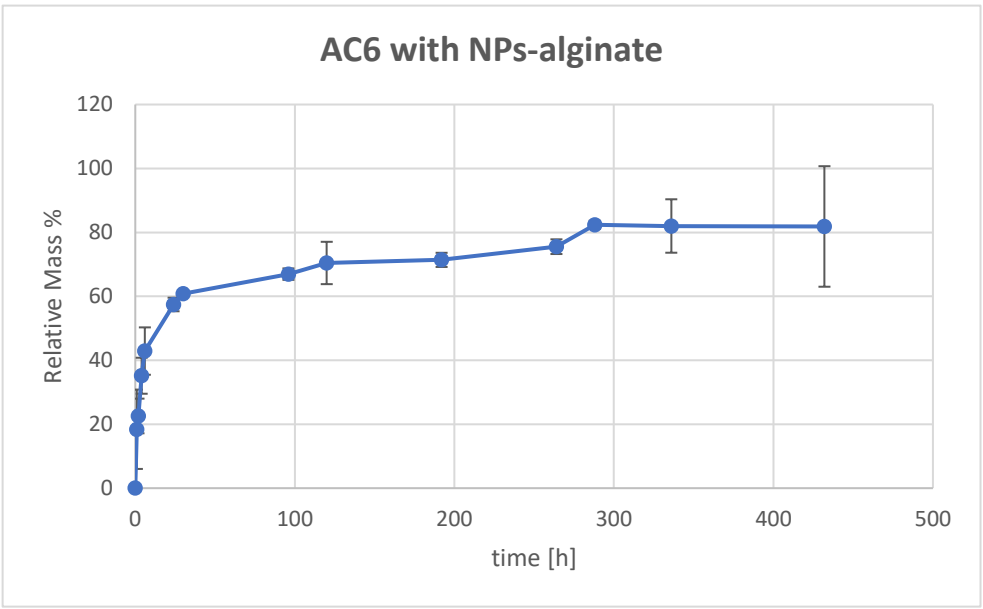

Figure 4.24: Release profile of NPs-alginate from AC6.

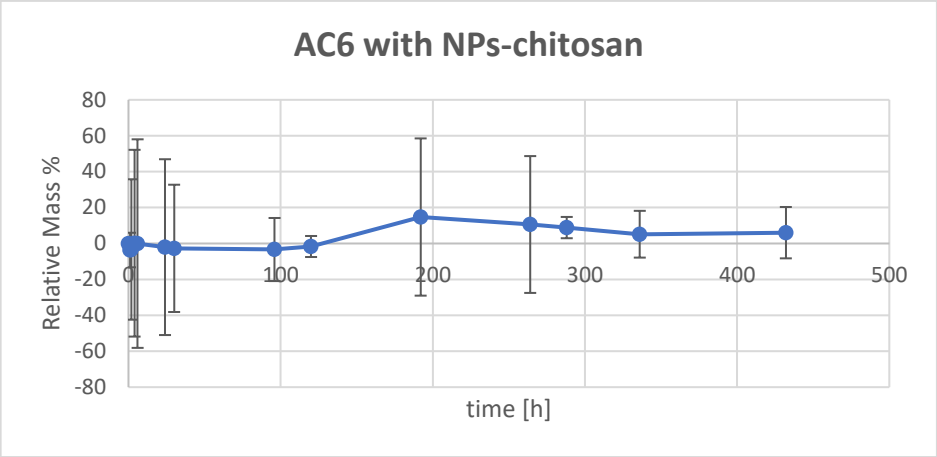

Figure 4.25: Release profile of NPs-chitosan from AC6.

As expected, the release kinetics are much slower, and the greater evidence for this fact is in figure 4.23, where the release of NPs-SDS is about 100% just after 120 h, with respect to the former 2 h. This is primarily because of the hydrogel mesh size that now is very tight.

Also, NPs-alginate, now halves their concentration in more than 24 h, with respect to the former profile, that sees a faster release, although the general behaviour is maintained unchanged, since the 10% of the payload is released in the first hour, and this can be the results of repulsive electrostatic interaction.

The same consideration can be done also for NPs-CTMAB, where a two-step profile is again evident and can be explained in the very same manner.

The real difference from the first analysed profiles, is the NPs-chitosan behaviour, that constitutes, actually, a great evidence of the difference between AC6 and AC1, that is the mesh size. Indeed, in this case it's possible to see that no significant release occurs, because the huge dimensions of NPs-chitosan (641.4 r.nm) are preventing the passage of nanoparticles through pores of hydrogel, that are order of magnitude smaller. In addition, NPs-chitosan have positive charge, so that the electrostatic attraction prevents the diffusion of nanoparticles in the supernatant solution.

4.3.3 Release profiles from AC6+CMC

In this case, the effective characterization of hydrogel is not present in literature, and this formulation is actually an innovation inside the biomedical field.

For this reason, all the possible considerations done on this kind of systems is obtained by inference from a direct comparison with the former hydrogel formulations.

In principle, the addiction of carboxymethyl cellulose inside the AC6 formulation leads to a decreasing of the ratio between hydroxyl and carboxyl groups (A/B), since CMC acts like another source of carboxyl groups. The consequence is that the mesh size of final hydrogel should be larger with respect to the classic AC6, since the cross-linking density is lower. On the other side, as far as concern electrostatic potential, the addition of CMC should lead to a greater anionic behaviour of the 3D matrix.

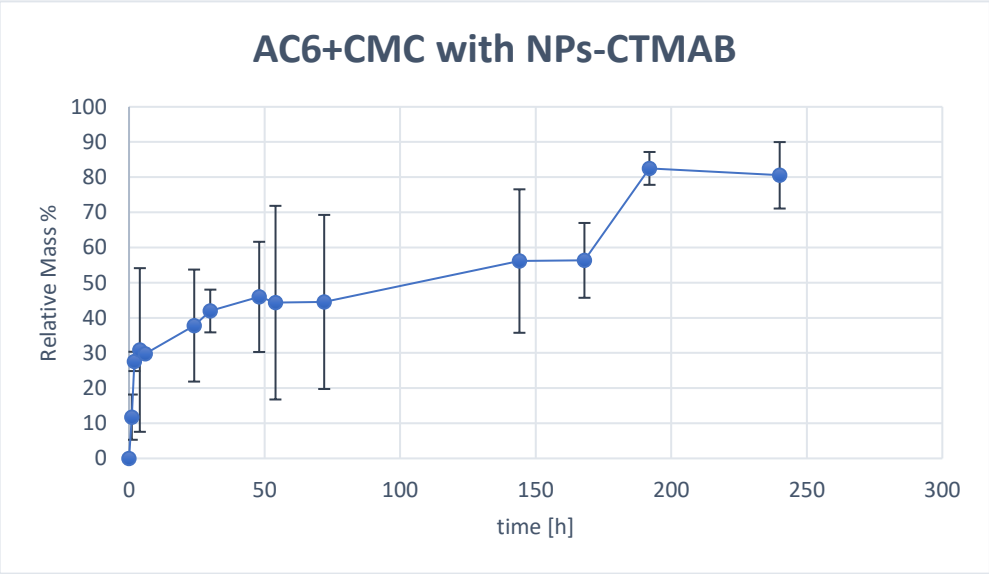

Figure 4.26: Release profile of NPs-CTMAB from AC6+CMC.

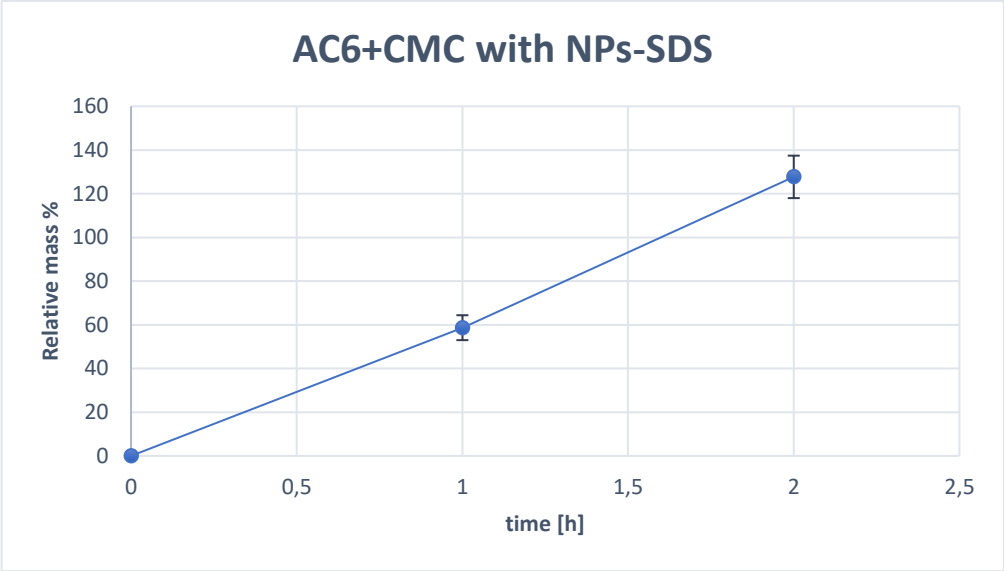

Figure 4.27: Release profile of NPs-SDS from AC6+CMC.

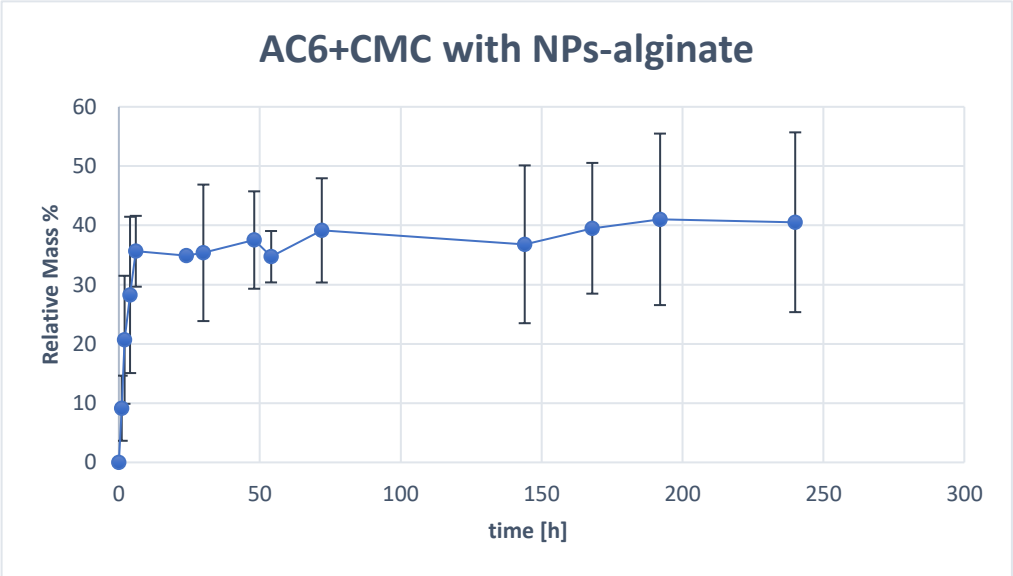

Figure 4.28: Release profile of NPs-alginate from AC6+CMC.

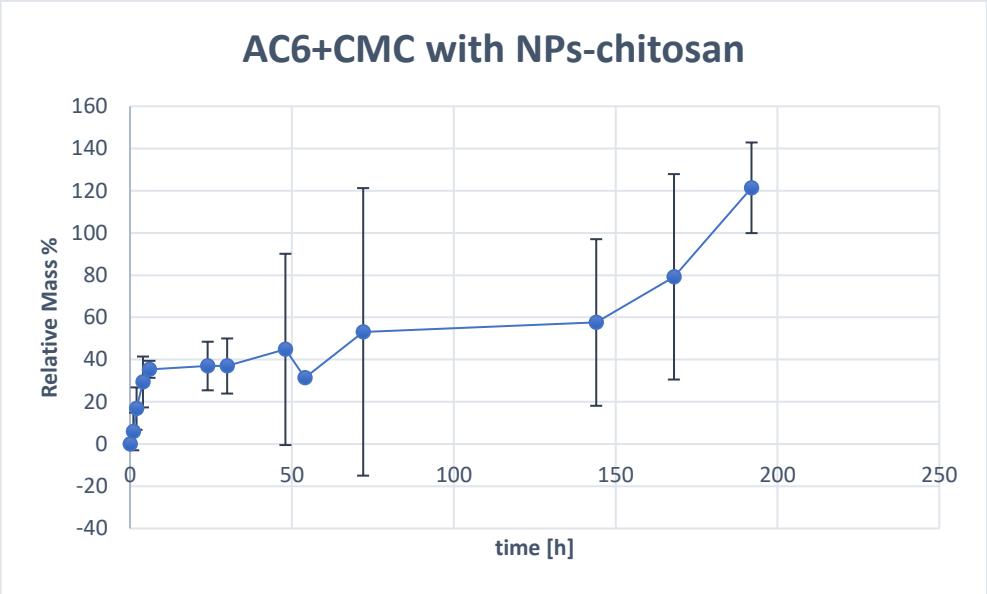

Figure 4.29: Release profile of NPs-chitosan from AC6+CMC.

The higher value of mesh size is proved by looking to the NPs-chitosan profile, that is more similar to that of AC1, since the A/B ratio has probably a value approximately similar to AC1's. Actually, a more detailed analysis shows two possibilities: the first considers that (i) the assumption of more negative network for AC6+CMC is not correct, because of some shielding effect on CMC charges, so that the attractive interaction between the NPs and AC6+CMC is weaker than that with AC1 and this results in a faster release of nanoparticles from AC6+CMC (50% release after 72 h for AC6+CMC with respect to about 155 h for AC1); in the second case, (ii) although the electrostatic attraction is higher, the mesh size is much larger that the release kinetic is faster.

More information can be obtained from NPs-SDS. In this case the release profile sees the halving of payload after more or less 1.55 h, whereas in AC1 it occurs at around 0.5 h. This can be an evidence that supports the expressed hypothesis of shielded charges of CMC, because if the interaction of two equal charges in AC6+CMC would have been stronger than AC1, the release should have been much faster.

Furthermore, the distance from the classic AC6 behaviour in terms of mesh size, is testified by the profile shape, that is typical of a pure diffusive release, that can be modelled with a simple Fick law, as it is for AC1.

A controversial trend is detected for NPs-alginate, that although should feel the more intense repulsive influence, since it is the more negative nanoparticle, with the hypothetic more negative hydrogel, it shows the slower release in comparison with both AC1 and AC6, since it just approaches the 50% of release.

On the way opposite, supporting the hypothesis of a A/B ratio in between the AC1's and AC6's, the release of NPs-CTMAB sees the halving of its payload at about 105 h, with respect to the AC1, that occurs at 54 h and AC6, that approaches this value after a long time and just because of the crumbling of the hydrogel matrix. This behaviour can actually be also in agreement with the theory of a greater negative charge of hydrogel chains, that promotes a slower release because of the higher attractive force, that make this profile in line with NPs-chitosan's.

In conclusion, the only way to clarify the real features of this system is to develop more specific physical, morphological and rheological studies to fully characterize this new hydrogel.

4.4 Nanocluster characterization

NPs electrostatic features have been exploited also for the assembling of nanoclusters, as resulting systems of the attractive interaction between opposite charged nanoparticles.

Exploiting the same four selected nanoparticles for the hydrogel releases studies, the nanocluster formation has been challenged contacting both NPs with an absorbed charged tenside and NPs with the polysaccharide functionalization.

Using NPs-CTMAB and NPs-SDS, different proportions have been tried as reported in table 4.7 below.

NPs-CTMAB	NPs-SDS	Z-average [r.nm]	PDI	ZP [mV]	St. Deviation [mv]
1	1	92.36	0.39	-34.7	10
2	1	106.5	0.45	-22.1	10.2
6	6	277	0.92	-27.8	12.9

Table 4.7: NPs-CTMAB and NPs-SDS nanocluster, an hour after synthesis.

Those analysis have been done just an hour after the nanocluster synthesis. It's possible to see that initially no evident increase of dimensions is seen; on contrary, it seems that polymeric particles have been subjected to shrinking, probably due to the electrostatic interaction with others. At the same time, a disparity of charge can be seen, since the zeta potential is revealing an anionic surface charge. However, those unforeseen results can be related to systems in non-equilibrium condition, where the nanoparticles are still subjected to a dynamic movement for the constitution of the final system.

Indeed, those analysis have been repeated after a week from synthesis, and more expected results have been obtained, as visible in table 4.8.

NPs-CTMAB	NPs-SDS	Z-average [r.nm]	PDI	ZP [mV]	St. Deviation [mv]
1	1	236.6	0.4	-7.68	3.81
2	1	136.5	0.51	-9.36	13.2
6	6	340.9	0.64	-12.1	10.3

Table 4.8: NPs-CTMAB and NPs-SDS nanocluster, a week after synthesis.

It can be clearly seen that the nanocluster formation is now more evident, since the dimensions of the system is increased with respect to both single nanoparticles and initial aggregation phase, although still in the nanoscale, thus good for biomedical applications. In addition, also the surface charge is now approaching to 0 value, due to the global balance between positive and negative charges.

The same approach has been adopted for NPs with polysaccharides functionalization. Different NPs amount have been used, as already descripted in paragraph 3.2. Results are reported after a day from synthesis in table 4.9 and after a week in table 4.10.

	Z-average [r.nm]	PDI	ZP [mV]	St. Deviation [mv]
CA37	769	0.35	-26.3	7.21
CA55	882	0.5	-29.6	12.2
CA73	540	0.38	29.9	6.24

Table 4.9: NPs-alginate and NPs-chitosan nanocluster, a day after synthesis.

	Z-average [r.nm]	PDI	ZP [mV]	St. Deviation [mv]
CA37	575.1	0.67	-19.8	5.54
CA55	846.2	0.6	-37.1	3.76
CA73	565	0.36	30.6	24.8

Table 4.10: NPs-alginate and NPs-chitosan nanocluster, a week after synthesis.

In this case a evident assemblage of particles is evident also from the initial contacting phase (figure 4.30) and this is proved also from data, since the average diameters are exceeding nanoscale also a day after the synthesis.

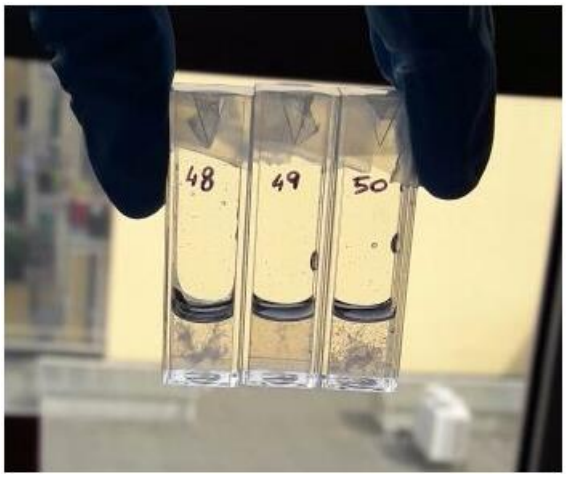

Figure 4.30: Cluster formation after contactment of NPs-alginate and NPs-chitosan in different ratio. They correspond to CA55, CA37 and CA73 respectively.

This is the main difference from these nanoparticles assembling and the former, dealing from tenside absorbed nanoclusters. Possibly, the influence of polysaccharide dimensions is affecting the final size of those clusters, but also the intensity of charges, that in this case are greater than the NPs-CTMAB and NPs-SDS'.

In addition to that, the surface charge now is not decreasing in time, so that after a week a certain amount of charge is always present. This is due to the disparity in charge intensity for NPs-chitosan (37 mV) and NPs-alginate (-54 mV). This difference becomes evident looking to the CA55 probe, where the concentration of positive and negative particles is the same. On contrary, inCA73, where the concentration of positive NPs-chitosan is 70%, the surface charge results positive.

Concluding, the nanocluster formation has been proved for both nanoparticles systems, one with the surface absorbed tenside and the other with the absorbed polysaccharides.

The ionic strength of those nanoparticles affects the final dimensions of cluster, since it tends to aggregate more units, but dimensions are obviously dependent also on the initial nanoparticle dimensions. Along with the average molecular weight of absorbed species influence it, that along with electrostatic stabilization, provide also steric stabilization, above all for the NPs-alginate and NPs-chitosan.

5 Conclusions

Drug delivery is the ensemble of approaches, formulation and systems used to achieve a therapeutic effect in humans or animals, transporting pharmaceuticals active agents to the pathological site, where they can express their therapeutic function.

Novel drug delivery systems are based on nanocarriers in order to obtain a controlled drug release. This necessity comes from the need to improve the classic drug administration routes, like oral, intravenous and intra-arterial ones. In particular, the main improvement would be the possibility to maintain drug concentration at an effective level for a determined period of time, avoiding under and over dosing. Several types of systems have been designed for this purpose, from macroscopic scaffolds to nanostructures.

Several diseases like spinal cord injury require treatments with multi target approach in order to have a local controlled delivery for both drugs and cells.

In this framework, a great deal of effort is now focusing on the engineering of composite hydrogel-nanoparticle systems, for their capability to combine in a synergic way the advantages of both nanoparticles and hydrogels. Indeed, they are biocompatible and biodegradable, flexible and versatile, since they lead to the possibility to load different drugs (both hydrophobic and hydrophilic), and to deliver them with specific controlled and prolonged kinetics *in situ*, avoiding systemic diffusion.

Polymers are appearing as the most promising materials for the obtainment of biodegradability and biocompatibility of composite systems. Both synthetic and natural polymers have been challenged and, finally the selected polymeric base to produce nanoparticles has been the PEG-PLA copolymer.

In order to get a composite system, electrostatic interactions between hydrogel and nanoparticles are exploited, thus different types of tenside, anionic and cationic, are exploited.

The nanoparticle synthesis has been realized with two different procedures, solvent diffusion method and solvent evaporation method. The former has been used for the synthesis of tenside coated NPs, where emulsifier is going to absorbe onto particle surface. From this preparation, NPs-SDS and NPs-CTMAB are obtained and subsequently characterized. On the other side, through solvent evaporation method, it has been possible to exploit polysaccharides as functionalization for nanoparticles surface: the absorption mechanism is the same as tenside but now alginate and chitosan are employed, getting NPs-chitosan and NPs-alginate, also characterized.

It has been found that all nanoparticles selected among all synthetized is suitable for the biomedical application and able to load hydrophobic drugs within the hydrophobic PLA core, since both PEG and tenside cooperate to stabilize the system.

Once nanoparticles have been obtained, the internalization into hydrogel is done, in order to study the release behaviour of nanoparticles in time and the effect of electrostatic interaction of those NPs with the hydrogel matrix on the delivery.

Different hydrogels have been challenged: AC1, AC6 and AC6+CMC. The former two formulations have already been studied deeply in literature, whereas the last one is an innovation in biomedical field and involves the same formulation of AC6 but with the introduction of the carboxymethyl cellulose, a natural polysaccharide added to increase the biocompatibility and biodegradability of this system.

It has been found that both electrostatic interaction and steric hindrance of nanoparticles compete into the release kinetics: AC1 and AC6, fully characterized have been useful to verify the internalization of nanoparticles and the actual delivery comparing different mesh sizes.

For AC6+CMC the treatment is similar, although the hydrogel characterization is not already available. However, through direct comparison with AC1 and AC6 it is possible to identify some features and have as idea of the electric charge of hydrogel chains and on the peculiar characteristics, such as mesh size, cross-linking density and hydroxyl, carboxyl groups ratio.

This research field is one of the further possible topics to be analysed, to create a new hydrogel formulation with more biocompatibility and biodegradability, able to be applied lonely or as a composite pharmaceutics vehicle with nanoparticles of different nature.

In addition to that, also the formation of nanoclusters has been assessed, as further application of the electric charge functionalization of nanoparticles. Electrostatic interactions between NPs of opposite charges has been used to create different systems, able to be applied in biomedical field as drug delivery systems.

Also in this area further development are possible, for example for the examination of the possibility to create nanogels of hydrogels via electrostatic interactions between aggregating nanoparticles.

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