**THE EFFICIENT DRUG BISPHOSPHONATES FOR MANAGEMENT OF BONE DISEASE OSTEOPOROSIS**

**Sandhya Pathak\*, Poonam Kohli, Chandni Pachouri and Archna Pandey**

 **Department of Chemistry, Dr. H. S. Gour Central University, Sagar (M.P), INDIA**

**Email:** sandhyapathak935@gmail.com (Corresponding Author)

**ABSTRACT**

[Osteoporosis](https://www.verywell.com/osteoporosis-4012671) is a bone-related disease with low Bone Mineral Density (BMD) than required. Bones become porous, more fragile and increase trouble of [bone](https://en.wikipedia.org/wiki/Bone_fracture) broking. The most affected bones are the spine, hip and shoulder. The main cause of Osteoporosis is decreasing BMD. The most effective and frequently used drugs for treatment of osteoporosis are different Bisphosphonate (BPs) molecules i.e. Alendronate, Ibandronate, Risedronate etc, due to the effective results of their therapeutic studies. The potency of BP’s for decreasing the risks of osteoporosis has been proved in many clinical studies.

The osteoclast cells are responsible for bone resorption and cause osteoporosis, so the bisphosphonate drugs target these cells and inhibit the osteoclast cells' activity. “Bisphosphonates bind with high affinity to the mineral matrix of the bone and prohibit osteoclast resorption of the bone, which result to a decrease in bone turnover and a net gain in bone mass. Risedronate ,Alendronate, zoledronic acid etc have evidence an increase in Bone mineral density and a lowers the risk of fractures due to osteoporosis in men and postmenopausal women.

Bisphosphonates bind to hydroxylapatite crystals i.e Ca5(PO4)3OH and therefore have a very high affinity for bone. Bisphosphonates are released from the bone matrix upon exposure to acid and enzymes secreted by an active osteoclast. Bisphosphonates bind the surface of bone and decline bone resorption of Osteoclast cells. The equilibrium or balance between osteoclast and osteoblast cells ceases the bone loss and increases bone strength, which is the basic action of Bispbospbonate class of drugs.

**Keywords:** Bisphosphonate,Bone mineral density (BMD), Bone resorption, Osteoclast, Osteoporosis

**INTRODUCTION**

[Osteoporosis](https://www.verywell.com/osteoporosis-4012671) means "Porous bone", a skeletal related disease with low Bone Mineral Density (BMD). Bones become more fragile and increase high chance of fracture. The most affected bones are the spine, pelvic, shoulder and wrist bones. Decreasing bone density is the leading cause of Osteoporosis. Pelvic and spinal fractures are the most risky aspect of Osteoporosis. It is commonly seen in old age, but women have a high risk after menopause. It causes Stooped posture, loss of height and persistent pain with cause’s reduction in movability [1].

Osteoporosis is a Greek word from “Osteen”, which means bone and “Porous” for porosity, indicating bone deterioration. Osteoporosis is sometimes confused with osteoarthritis because the name is similar. Osteoporosis is a skeletal-related bone disease, whereas osteoarthritis is a disease of the joints and surrounding tissues**.**

Osteoporosis is one of the most common bone diseases diagnosed by low BMD and fragility of bone tissues with a subsequent increase in sensitivity to fracture. It is called a “Silent disease” because its symptoms cannot identify at an early stage which caused the fractures. Osteoporotic fractures often increase mortality, reduce life quality, prolonged hospital stays and high economic costs [2, 3]. It is generally seen in senior citizen, but women after menopause have a high risk of Post- Menopausal Osteoporosis (PMO). On Compared with the myocardial restriction and stroke, they need nursing home admissions and long duration stay after osteoporotic pelvic fractures [4].

According to the World Health Organization (WHO) definition, 26 million white Americans are osteoporotic. According to the 2011 census, approximately 183 million Indians are above 50. Years are osteoporotic; this number is expected to double by 2025. Approximately 40% of patients suffering from a hip fracture cannot live independently, and 22% die within 12 months of the fracture [5, 6].

Osteoporosis is designate by a decreases in mass of bone also known as Osteopenia and a deterioration in bone micro-architecture, leading to an increase softness of the skeleton and, therefore, to a greater risk of bone cracking. Postmenopausal osteoporosis is also a serious public health problem due to its worldwide universality and the high socio-economic and healthcare influence on society [7].

India is the second-most populous country globally, so osteoporosis is a severe problem in our country. The population of India is anticipated to increase to 1,367 million by 2021 and 1,613 million by 2050, of which 9% (134 million) and 19.6% (315 million), respectively, will be adults over 60 years [8].

 **CURRENT TREATMENTS FOR OSTEOPOROSIS**

Common osteoporosis treatments are changing lifestyles (including exercise and meditation in everyday life), taking orthopedic drugs, and surgical treatment. [Biphosphonate medications](https://en.wikipedia.org/wiki/Bisphosphonate%22%20%5Ct%20%22_blank) are mainly used to treat previous broken bones due to osteoporosis. “Osteoporosis is a second worldwide disease after cardiovascular diseases”. It causes more than 9 million fractures per year. Therefore researchers have turned to a new branch of nanotechnology, i.e. nanomedicines, to create alternative and innovative treatments for osteoporosis [9].

“In terms of treatment choice for osteoporosis, the National Osteoporosis Foundation (NOF) suggests starting with a non-pharmacologic approach. Resistance and weight-bearing exercise can increase muscle mass and rapidly increase Bone mineral density. Yoga raise balance and increase muscle tone, which as a secondary effect decrease the risk for falls among mature or aged patients. Recommending about smoking as well as alcohol cessation (which is directly linked to reduce BMD) are encouraged” [10-12].

Recent treatment of osteoporosis involves bisphosphonates, Denosumab, calcitonin, Selective Estrogen Receptor Modulators (SERM), i.e. Raloxifene and sufficient consumption of calcium along with vitamin D. Novel osteoclast targeted agents like c-src kinase and cathepsin K are under medical advancement[13, 14].

Bisphosphonates are the most frequently used drugs for curing osteoporosis due to the positive and better results of their medicinal studies. The efficacy of BP’s for reducing the possibility of osteoporosis has been established in large clinical trials; the target cells of bisphosphonates are Osteoclasts . BP’s are the most powerful active drugs among all available drugs for the treatment of osteoporosis. They are chemically derived from pyrophosphates that suppress the precipitation of calcium carbonate [15].

B Akgun and co-workers have described the Synthesis and analysis the Chemical structure of pyrophosphate and bisphosphonate. R1 and R2 indicate the side chains of bisphosphonate Fig. (1).

 **Figure (1) Chemical structure of BPs**

Bisphosphonates are synthetic analogs with a P–C–P bond in place of the P–O–P bond of inorganic pyrophosphates. Strong binding affinity to bone is the unique characteristic of bisphosphonates which is used to cure bone resorption and other bone-related diseases. BP’s prohibit the calcification and breakdown of Bone minerals, i.e. hydroxyapatite, which are bound by two phosphate unit and serve as“Bone hook” [16].

Bisphosphonates when attached the bone surface and decline bone resorption of Osteoclast cells. Balance between osteoclast and osteoblast cells stopped bone loss and enhance bone strength, which is the primary action of Bispbospbonate class of drugs.



**BONE MINERAL DENSITY (BMD)**

Bone density values in individuals can be indicate concerning a reference population in standard deviation (SD); when it compared to the youngster , healthy population, this measurement is referred to as the T-score [17-19]. The correlation of BMD and T score is shown in Fig. (2).

**Figure (2) Correlation of BMD and T-score**

Osteoporosis: T-Score 2.5 SD or more below is called osteoporosis. Acute osteoporosis: T-Score 2.5 SD or more below in the presence of one or more fragility fractures (T-score d”-2.5 PLUS fracture).

**DIAGNOSIS OF OSTEOPOROSIS**

According to World Health Organization (WHO), “The most exact diagnostic technique for osteoporosis is the measurement of BMD by Dual Energy X-ray Absorptiometry (DEXA), but it is expensive and not universally available in low economies” [20].

In this disease, the following symptoms are observed:-

1. Fragility of bone tissue
2. Low bone mass (BMD)
3. Back pain caused by collapsed vertebra or fracture
4. A stooped posture

These symptoms increased the risk of fractures. According to WHO report, the standard range of BMD for osteoporotic hip and spinal bones is 2.5 as measured by DEXA technique. The reference values of BMD of healthy adults were calculated and termed as “T-score value”. If this value for spinal or hip BMD is between 1 and 2.5 and standard deviations below the mean is defined as “Osteopenia”. If people have osteopenia, they are at the high risk of osteoporosis [21]. The World Health Organization has established the following diagnostic guidelines shown below in the table (1):-

**Table 1. -Standard** [**T-score**](https://en.wikipedia.org/wiki/Bone_density#T-score) **range**

|  |  |  |
| --- | --- | --- |
|  **Osteoporosis Category** | [**T-score**](https://en.wikipedia.org/wiki/Bone_density#T-score) **Value** | **% young women** |
| 1. Normal
 | [T-score](https://en.wikipedia.org/wiki/Bone_density#T-score) ≥ −1.0 | 85% |
| 1. Osteopenia
 | 2.5 < T-score < −1.0 | 14% |
| 1. Osteoporosis
 | T-score ≤ −2.5 | 0.6% |
| 1. Severe osteoporosis
 | T-score ≤−2.5 with fragility fracture  | 0.4% |

Radio graphical methods are applied for the diagnosis of Osteoporosis. DEXA scanning is the most commonly used diagnosis technique for osteoporosis in central of the total hip, neck of femur or lumbar spine. Quantitative determination of BMD is possible with the help of Computed tomography (CT scan). It is the most frequently used technique, but its use is confined due to radiation exposure and cost.

**BISPHOSPHONATES**

The most frequently used drugs for the treatment of osteoporosis are Bisphosphonates due to a the efficacy and minimizing the risks of osteoporosis, has been established in many clinical trials. Zoledronic acid, having nitrogen belongs to bisphosphonate group, has been proved the potent drug for the management of osteoporosis and lowers the risk of fracture.

 According to the mechanism of Bisphosphonates, bind with great affinity to the bone mineral matrix (Hydroxy apetite) and target the osteoclast bone cells. Bisphonates prevent osteoclast resorption of the bone, resulting to a decrease in bone turnover and increase the BMD.  Alendronate, risedronate, and zoledronic acid have reported to increase in BMD and a decrease the bone resorption. It helps in treatment of osteoporosis in men and postmenopausal women [22].

Bisphosphonates are mostly prescribed drugs for the treatment of osteoporosis in the US and many more countries, including India. Alendronate is the first bisphosphonate, which was authorized in the US in 1995, for the treatment of osteoporosis.

Since then, many new bisphosphonates molecules with short frequent dosing intervals have been introduced.

1. Risedronate is an oral medication administered daily, weekly as well as monthly at varying doses.
2. Zoledronic acid is the newer medication administered once yearly by intravenous transfusion.

Bisphosphonates bind to hydroxyapatite crystals and target the bone cells osteoclast with great affinity [23]. Bisphosphonates are released from the bone matrix upon exposure to acid and enzymes secreted by an active osteoclast. Out of all bisphosphonates, zoledronic acid has the highest affinity for binding to the bone mineral matrix followed by the following order [24], shown below in fig.(3).

**Pamidronate >Alendronate > Ibandronate > Risedronate > Etidronate > Clodronate**.

The the targetability of bisphosphonate for the bone matrix, phosphate and hydroxyl groups are essential. The potency of bisphosphonates for the inhibition of bone resorption depends on the structural moiety (in the R2 position) bound to the central carbon atom. The bisphosphonate’s antiresorptive potency can be increased by addition of nitrogen or amino group up to 1000 times relative to early non–nitrogen-containing bisphosphonates, such as etidronate. Recent studies explain The molecular mechanism of inhibition of osteoclast activity of nitrogen-containing bisphosphonates has been explained in many studies.

High affinity to bone minerals is the most important pharmacological feature of all bisphosphonates, which makes these molecules, highly effective in orthopaedic therapy. This high affinity for bone minerals allows bisphosphonates to achieve a high local concentration throughout the entire skeleton. Accordingly, bisphosphonates have become the primary therapy for skeletal disorders characterized by excessive or imbalanced skeletal remodelling, in which osteoclast and osteoblast activities are not tightly coupled, leading to excessive osteoclast-mediated bone resorption [25-31].



**Figure (3) Efficacy of Bisphosphonates**

Etidronate, clodronate, and tiludronate are non–nitrogen-containing bisphosphonates, considered first generation bisphosphonates. Alendronate, risedronate, ibandronate, pamidronate, and zoledronic acid are second and third generation bisphosphonates have nitrogen-containing R2 side chains [32]. Classification of bisphosphonates based on nitrogen-containing and non-nitrogen containing groups are shown in the following Fig. (4).



**Figure (4) Classification of Bisphosphonates**

Zoledronic acid is the most efficient bisphosphonate molecule, with higher affinity to bone matrix binds to the bone surface but spread through bone slowly, whereas some lower affinity bisphophonate molecule like clodronate distributes more widely through the bone, but they have a shorter time of residence when the treatment is stopped. Approximately three months of oral bisphosphonate therapy can suppress the bone resorption with regardless of dosing frequency, but it is more efficient in case of intravenous administration [33, 34].

**COMMOM BISPHOAPSPHONATES**

1. **Alendronate –Sodium [35, 36]**

**IUPAC Name :** Sodium trihydrate hydrogen (4-amino-1-hydroxy-1- phosphonobutyl) phosphonate

****

**Structure :**

**Molecular Formula** : [C4H12NNaO7P2](https://pubchem.ncbi.nlm.nih.gov/#query=C4H12NNaO7P2)

**Molecular Weight** : 271.08

**Physical State**  : Solid, White, non hygroscopic crystalline powder

**Solubility**  : Soluble in water, very slightly soluble in alcohol

**Pharmacological Class** :Alendronate-sodium is a second generation bisphosphonate which was first time approved by FDA for the treatment of of osteoperosis. It prevents the resorption of bone [label](https://go.drugbank.com/salts/DBSALT000315#label-reference).

**Melting Point :** 234ºC

**Mechanism of action**

Alendronate binds to bone mineral hydroxyapatite and targets the osteoclasr cells which are responsible for bone resorption. It causes local acidification, releasing alendronic acid taken into osteoclasts by fluid-phase endocytosis. Endocytic vesicles are acidified, releasing alendronic acid to the cytosol of osteoclasts, where they induce apoptosis. It inhibits the activity of osteoclasts cells, which decreases bone resorption and is shown through decreased urinary calcium.

**(ii) Ibandronate –Sodium**

**IUPAC Name :** 1-Hydroxy-3-[methyl (pentyl) amino]-1, 1- propanediyl} bis (phosphonic acid)

**Structure :**

**Molecular Formula :** C9H24NNaO8P2

**Molecular Weight :** 359.23

**Physical State :**  Solid, White to off white powder

**Solubility :** Freely soluble in water, practically insoluble in organic solvents.

**Melting Point :** 173-175°C

**Mechanism of Action**

Ibandronate is Nitrogen-containing bisphosphonate taken into the bone and binds to the hydroxyapatite. Bone resorption by osteoclasts causes local acidification, releasing the bisphosphonate, which is taken into the osteoclast by fluid-phase endocytosis. Endocytic vesicles become acidified, releasing bisphosphonates into the cytosol of osteoclasts where they act. Polymeric nanoformulation and nanoencapsulation of bisphoaphonate drugs can improve the oral bioavailability of many drugs, have been proved a promising approach in previous studies [37-39].

**(iii) Risedronate –Sodium**

**IUPAC Name :** Sodium hydrogen [1-hydroxy-1-phosphono-2-(pyridin-3- yl)ethyl]phosphonate

**Structure :**

**Molecular Formula :**  C7H10NNaO7P2

**Molecular Weight :** 305.0941

**Physical State :**  Solid, Off white crystalline powder.

**Solubility :** Soluble in pH 7.0 potassium phosphate dibasic

solution, 0.1 N NaOH, and water; very slightly soluble in 0.1 N HCl, practically insoluble in C2H5OH, and insoluble in isopropanol.

 **Melting Point :** 252-262 ºC

**Mechanism Of Action**

Risedronatic acid targets to bone hydroxyapatite [Label](https://go.drugbank.com/drugs/DB00884#label-reference). Bone resorption causes local acidification, releasing risedronic acid, which is taken into osteoclasts by fluid-phase endocytosis[1](https://go.drugbank.com/drugs/DB00884#reference-A959). Endocytic vesicles are acidified, releasing risedronic acid to the cytosol of osteoclasts, where they induce apoptosis through inhibition of farnesyl pyrophosphate synthase[1](https://go.drugbank.com/drugs/DB00884#reference-A959). It inhibits the activity of osteoclasts and decreased the bone resorption [40].

**CONCLUSION**

Osteoporosis is a common worldwide disease after cardiovascular disease. Many medications are available for the treatment of osteoporosis but bisphosphonate molecules are the most efficient medication option, Bisphosphonates are the most effective treatment option for Osteoporosis, but there is increasing concern about their long-term safety. The Bisphosphonates are the most commonly prescribed drugs by FDA to treat osteoporosis. Alendronate was the first bisphosphonate to be approved to treat osteoporosis in the US. Since then, newer bisphosphonates with less frequent dosing intervals have been introduced like Risedronate, Zoledronic acid etc. Bisphosphonates bind to hydroxyapatite crystals and thus have a very high affinity for bone.

The review suggested the different alternatives available for the treatment of osteoporosis. The assessment was specially centered at the remedy to osteoporosis & different bone associated diseases. The biphosphonates treatment minimized the bone problems including physiotherapy, surgeries, etc. So the review article includes all the general information regarding biphosphonates. These are the drug of desire that's effortlessly to be had with nicely tolerance to patients & now no longer having life-threatening facet effects.

**FUNDING**

Nil

**AUTHORS CONTRIBUTIONS**

The authors have contributed equally.

**CONFLICT OF INTERESTS**

The authors declare no conflicts of interest.

**REFERENCES**

1. Kawalkar AC. "A comprehensive review on osteoporosis." J Trauma, 2015, 10: 3-12.
2. Golob AL, Laya MB. Osteoporosis: screening, prevention, and management. Medical Clinics. 2015 May 1;99(3):587-606.
3. WHO Study Group on Assessment of Fracture Risk, and its Application to Screening for Postmenopausal Osteoporosis. Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. No. 843-849. World Health Organization, 1994.
4. Rapp K, Rothenbacher D, Magaziner J, Becker C, Benzinger P, König HH, Jaensch A, Büchele G. Risk of nursing home admission after femoral fracture compared with stroke, myocardial infarction, and pneumonia. Journal of the American Medical Directors Association. 2015 Aug 1; 16(8):715-e7.
5. Daroszewska A. Prevention and treatment of osteoporosis in women: an update. Obstetrics, Gynaecology & Reproductive Medicine. 2012 Jun 1;22(6):162-9.
6. Malhotra N, Mithal A. Osteoporosis in Indians. Indian Journal of medical research. 2008 Mar 1;127(3).
7. Brown JP, Josse RG, Scientific Advisory Council of the Osteoporosis Society of Canada. 2002 clinical practice guidelines for the diagnosis and management of osteoporosis in Canada. Cmaj. 2002 Nov 12;167(10 suppl):S1-34.
8. Luy MA, classification of the nature of mortality data underlying the estimates for the 2004 and 2006 United Nations’ World Population Prospects. Comparative Population Studies. 2010;35(2).
9. Garriguet D. Bone health: osteoporosis, calcium and vitamin D. Health reports. 2011, 22(3) 7.
10. Khajuria DK, Razdan R, Mahapatra DR. Drugs for the management of osteoporosis: a review. Revista brasileira de reumatologia. 2011; 51:372-82.
11. Hinton PS, Nigh P, Thyfault J. Effectiveness of resistance training or jumping-exercise to increase bone mineral density in men with low bone mass: A 12-month randomized, clinical trial. Bone. 2015,79: 203-12.
12. Black DM, Rosen CJ. Postmenopausal osteoporosis. New England Journal of Medicine. 2016; 374(3):254-62.
13. Dhaliwala R, et al. The relationship of Physical performance and Osteoporosis prevention with vitamin D in older African Americans (PODA); Contemporary Clinical Trials, 2018; 65: 39–45.
14. Weaver CM, Alexander DD, Boushey CJ, Dawson-Hughes B, Lappe JM, LeBoff MS, Liu S, Looker AC, Wallace TC, Wang DD. Calcium plus vitamin D supplementation and risk of fractures: an updated meta-analysis from the National Osteoporosis Foundation. Osteoporosis International. 2016 27(1): 367-76.
15. De Luca L, Chiminazzo A, Sperni L, Strukul G, Scarso A. Pyrrolidine‐Containing Bisphosphonates as Potential Anti‐Resorption Bone Drugs. Chemistry–A European Journal. 2017 Mar 8;23(14):3474-8.
16. Akgun B, Avci D. Synthesis and evaluations of bisphosphonate‐containing monomers for dental materials. Journal of Polymer Science Part A: Polymer Chemistry. 2012 Dec 1;50(23):4854-63.
17. Asia-Pacific Regional Audit (2013) Epidemiology, costs and burden of osteoporosis in 2013.International Osteoporosis Foundation, 2017.
18. Bukhari M. The National Osteoporosis Guideline Group's new guidelines: what is new?. Rheumatology. 2009, 48(4):327-9.
19. Kanis JA, Johnell O, Odén A, Johansson H, McCloskey EF. FRAX™ and the assessment of fracture probability in men and women from the UK. Osteoporosis international. 2008, 19(4):385-97.
20. Guglielmi G, Scalzo G. Imaging tools transform diagnosis of osteoporosis. Diagnostic Imaging Europe. 2010, 26(3):7-11.
21. Kanis JA. "Osteoporosis." Journal of Medical Sciences 3(3), (2010):124-130.
22. Coxon FP, Thompson K, Rogers MJ. Recent advances in understanding the mechanism of action of bisphosphonates. Current opinion in pharmacology. 2006 Jun 1;6(3):307-12.
23. Nancollas GH, Tang R, Phipps RJ, Henneman Z, Gulde S, Wu W, Mangood A, Russell RG, Ebetino FH. Novel insights into actions of bisphosphonates on bone: differences in interactions with hydroxyapatite. Bone. 2006 May 1;38(5):617-27.
24. Leu CT, Luegmayr E, Freedman LP, Rodan GA, Reszka AA. Relative binding affinities of bisphosphonates for human bone and relationship to antiresorptive efficacy. Bone. 2006 May 1;38(5):628-36.
25. Fosamax (alendronate sodium) prescribing information. Whitehouse Station, New Jersey: Merck; 2016.
26. Actonel (risedronate sodium) prescribing information. Rockaway, New Jersey: Warner Chilcott, LLC; 2015.
27. Atelvia (risedronate sodium) prescribing information. North Norwich, New York: Warner Chilcott, LLC; 2015.
28. Boniva tablets (ibandronate) prescribing information. South San Francisco, California: Genentech USA, Inc; 2016.
29. Binosto (alendronate sodium) prescribing information. San Antonio, Texas: Mission Pharmacal Co; 2015.
30. Boniva injection (ibandronate) prescribing information. South San Francisco, California: Genentech USA, Inc; 2015.
31. Reclast (zoledronic acid) prescribing information. East Hanover, New Jersey: Novartis Pharmaceuticals Corporation; 2016.
32. Kavanagh KL, Guo K, Dunford JE, Wu X, Knapp S, Ebetino FH, Rogers MJ, Russell RG, Oppermann U. The molecular mechanism of nitrogen-containing bisphosphonates as antiosteoporosis drugs. Proceedings of the National Academy of Sciences. 2006;103(20):7829-34.
33. Giger EV, Castagner B, Leroux JC. Biomedical applications of bisphosphonates. Journal of Controlled Release. 2013;167(2):175-88.
34. Lee D, Heo DN, Kim HJ, Ko WK, Lee SJ, Heo M, Bang JB, Lee JB, Hwang DS, Do SH, Kwon IK. Inhibition of osteoclast differentiation and bone resorption by bisphosphonate-conjugated gold nanoparticles. Scientific reports. 2016 Jun 2;6(1):1-1.
35. Jahnke W, Henry C: An in vitro assay to measure targeted drug delivery to bone mineral. ChemMedChem. 2010 May 3;5(5):770-6.
36. Nancollas GH, Tang R, Phipps RJ, Henneman Z, Gulde S, Wu W, Mangood A, Russell RG, Ebetino FH: Novel insights into actions of bisphosphonates on bone: differences in interactions with hydroxyapatite. Bone. 2006 May;38(5):617-27.
37. Russell RG, Watts NB, Ebetino FH, Rogers MJ: Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy. Osteoporos Int. 2008 19(6):733-59.
38. Sacco P, Brun F, Donati I, et al.  On the correlation between the microscopic structure and properties of phosphate-cross-linked chitosan gels. ACS Appl Mater Interfaces 2018.
39. Iswanti FC, et al. Preparation, characterization, and evaluation of chitosan-based nanoparticles as CpG ODN carriers. Biotechnology and Biotechnological equipment 2019; 33(1): 390–396.
40. Elkady OA, Tadros MI, El-Laithy HM  [QbD Approach for Novel Crosslinker-Free Ionotropic Gelation of Risedronate Sodium-Chitosan Nebulizable Microspheres: Optimization and Characterization.](https://pubmed.ncbi.nlm.nih.gov/31807950/), AAPS PharmSciTech. 2019, 21(1):14.