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Article

# Higher pH level increases the efficacy of calcium phosphate-mediated intracellular delivery

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## SUMMARY

**The entry of drugs into cells is crucial to their effectiveness because most drugs exert their therapeutic effects intracellularly. Cells are enclosed by the plasma membrane, which consists of lipids, proteins, and a glycocalyx and serves as a protective barrier limiting drug entry into cells. Changes in the external environment can alter the properties of the membrane, affecting the uptake of extracellular molecules. Among various factors, the pH of the cellular environment substantially influences the properties of the plasma membrane and drug ionization, the process by which a drug gains or loses an electric charge in response to the pH change. Calcium phosphate is commonly used as a filler for drug formulations. To date, a few studies have been done on the effect of pH levels on the efficiency of calcium phosphate for drug delivery. A lower pH increases the $Ca^{2+}/PO_4^{3-}$ ratio, leading to a higher positive charge. Consequently, we hypothesized that a lower pH could enhance the interaction of calcium phosphate with the negatively charged cell membrane, thus facilitating drug delivery into cells. To test this hypothesis, we used fluorescein as a model molecule for delivery and examined the impact of various pH levels on delivery efficiency. Surprisingly, we found that the efficacy of the delivery increased with higher pH levels. Although the results contradicted our initial hypothesis based on the chemical properties of calcium phosphate, our findings underscore the complexity of intracellular drug delivery, wherein changes in pH levels influence drug transport.**

## INTRODUCTION

The effectiveness of a drug is closely associated with its uptake efficiency, as most drugs deliver therapeutic effects once inside cells (1). A key component of uptake efficiency is the ability of drugs to pass through the plasma membrane. This lipid bilayer, approximately 4–10 nanometers thick, is composed of phospholipids and cholesterol that encloses cells (2, 3). The membrane is crucial for providing structural integrity, maintaining fluidity, and facilitating various cellular processes. Embedded within the lipid bilayer are proteins that function in cell communication and signaling pathways, regulate passage of molecules, and mediate cell-cell interactions (4). Additionally, lipids and proteins are frequently attached to polysaccharides to form the glycocalyx, a carbohydrate-rich layer on the cell surface that helps maintain cell integrity (5). All three major components of the plasma membrane work in concert to maintain cellular function and homeostasis (6).

Since the plasma membrane serves as the barrier between the intracellular and the external environment, its properties control the entry of extracellular molecules (4). Among various factors, including temperature and osmolarity, the pH of the cell's external environment significantly influences the properties of the plasma membrane (7). In addition, variations in pH can directly affect the ionization of drugs, altering their solubility and their ability to traverse biological membranes (8). For example, changes in pH can alter the protonation status of ionizable functional groups on drug molecules, affecting their interactions with membrane transporters and channels (9).

For drug formulation, chemical inertness and compatibility with most other pharmaceutical components are critical properties, making calcium phosphate one of the most commonly used excipient vehicles for the main therapeutic ingredient (10). Calcium phosphate can act as a binder or filler to maintain the drug integrity during the manufacturing process and ensure proper breakdown in the digestive tract (11). In addition, positively charged calcium ions from calcium phosphate interact with negatively charged molecules on cell membranes, facilitating their delivery into cells (12). Furthermore, calcium phosphate also exhibits physical properties that enable efficient mixing, compression, and tablet formation. Taken together, calcium phosphate has favorable chemical properties that enhance drug delivery, stability, and manufacturing, ensuring an efficient and controlled release of medications within the body.

To assess the impact of pH on the uptake of small molecule drugs, we chose to use fluorescein as a model molecule in our study. Fluorescein is one of the compounds widely used in cell experiments and drug delivery studies (13). The molecular weight of fluorescein is approximately 332.31 g/mol, which falls within the range of 100 to 900 g/mol for typical small molecule drugs (14). Fluorescein can be employed as a fluorescent tracer in living cells due to its minimal cell-modulatory or cytotoxic effects and high compatibility with physiological cell conditions (15, 16). Notably, fluorescein is typically a negatively charged molecule due to carboxylic acid groups that can ionize in aqueous solutions (16). Given that the plasma membrane is also negatively charged, this property impedes fluorescein from passing through the membrane on its own.

In this study, we aimed to investigate the impact of pH levels on intracellular molecule delivery using a calcium phosphate coupled fluorescein model. Based on the rationale that a lower pH elevates the $Ca^{2+}/PO_4^{3-}$ ratio, resulting in an increased positive charge, we hypothesized that lower

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pH could enhance the interaction of calcium phosphate with the cell membrane, thus facilitating drug delivery into cells. Surprisingly, we observed the opposite result: the effectiveness of fluorescein delivery increased with higher pH levels.

## RESULTS

To determine whether pH levels affect the efficacy of fluorescein delivery by calcium phosphate, we used Caco-2 cells, a colon tissue cell line commonly used to study intestinal absorption and prepared Minimum Essential Medium (MEM) containing calcium phosphate and fluorescein at pH levels of 6.8, 7.0, and 7.2 (17). Unlike Dulbecco's Modified Eagle's Medium (DMEM), the medium typically used for Caco-2 cell culture, MEM does not have a buffering system, making it easier to adjust its pH. However, given that Caco-2 cell growth is sub-optimal in MEM, we only incubated cells with our experimental MEM pH media for six hours. For control samples, similar media was used without fluorescein to examine any calcium phosphate autofluorescence at these pH levels. We also included a negative control by adding fluorescein alone to cells cultured in DMEM at 7 to determine whether fluorescein could enter cells without calcium phosphate.

We prepared three replicate samples for each experimental condition and captured three microscopy images using both bright-field and fluorescent filters. We found that while no fluorescence was observed in control samples, fluorescence intensities were highest in cells cultured in media containing calcium phosphate and fluorescein at pH 7.2, compared to pH 7.0 and 6.8 (**Figure 1**).

Next, to quantitatively assess fluorescein uptake, we counted the number of cells from bright-field images and assessed the cells' fluorescence intensity from the fluorescence images using ImageJ. We then calculated the fluorescence intensity per cell by dividing the total fluorescence intensity by the number of cells. We found that fluorescence intensities were significantly higher in cells cultured in calcium phosphate/fluorescein-containing media at pH 7.2 and pH 7.0, compared to pH 6.8 ($p<0.05$, **Figure 2**). We did not find any significant difference between fluorescence of cells cultured at pH 7.2 and pH 7.0 ($p=0.75$). We did not detect any fluorescent signal in any condition without fluorescein, suggesting that the fluorescence was due to fluorescein uptake into cells. We also trypsinized cells cultured in our experimental media conditions and counted them to assess cell viability. We confirmed that Caco-2 cells cultured in MEM with altered pH for 6 hours did not show significant cell death, maintaining an average cell viability of approximately 95% (**Figure 3**). These results affirmed that the fluorescence detected was not due to autofluorescence from dying cells. Taken together, these findings suggested that calcium phosphate-based fluorescein delivery was most effective at pH 7.2 compared to 6.8.

## DISCUSSION

In this study, we investigated the effect of pH on calcium phosphate-mediated intracellular drug delivery using fluorescein as a model compound. Although we hypothesized that pH 6.8 would result in the highest intracellular fluorescein levels, we found the opposite result: pH 7.2 had the highest intracellular delivery. This finding suggests greater complexity

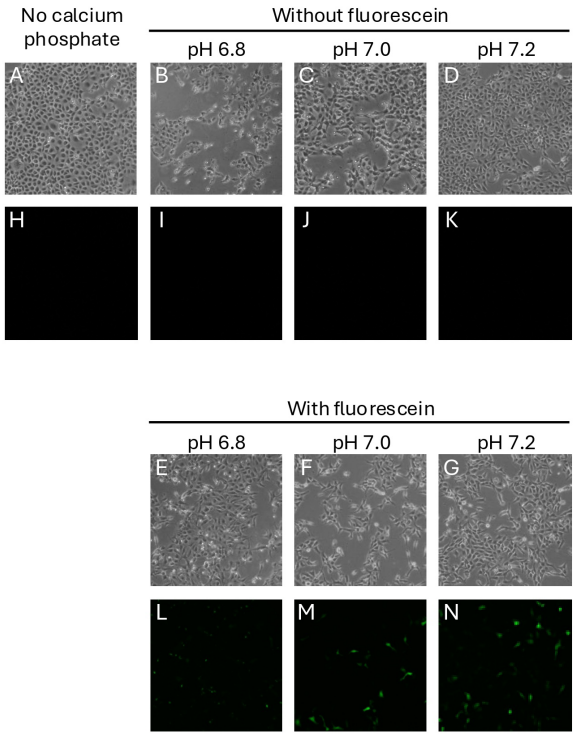


**Figure 1: Calcium phosphate-mediated intracellular delivery of fluorescein is most effective at pH 7.2 compared to pH 7.0 and 6.8.** For control groups, Caco-2 cells were cultured in medium containing 100 nM fluorescein without calcium phosphate (A, H) or in the presence of 0.1 M calcium phosphate at pH levels of 6.8 (B, I), 7.0 (C, J), and 7.2 (D, K) without fluorescein for 6 hours. For experimental groups, Caco-2 cells were cultured in medium containing 0.1 M calcium phosphate and 100 nM fluorescein at pH levels of 6.8 (E, L), 7.0 (F, M), and 7.2 (G, N) for 6 hours. Cell images were captured using both bright-field (A-G) and fluorescent filters (H-N). Three replicate samples were prepared for each experimental condition, and three microscopy images were captured for each replicate. Representative images of three replicates of each condition at 100X magnification are shown.

involved in intracellular delivery processes and highlights the need for further investigation of the interaction between pH dynamics and small molecule drug delivery.

In fact, the activity of cell surface transporters for small molecules, including fluorescein, are known to be altered by changes in pH. For example, the monocarboxylate transporter (MCT), a proton co-transporter located in neural, intestinal, and epithelial cells, has been shown to regulate intracellular fluorescein uptake (18-21). MCT-mediated transport is pH-dependent, with a decrease in pH reducing the ability of MCT to export anions and compromising its capacity for transporting molecules, consistent with our findings (22). Beyond transporters, the pH of the microenvironment can also influence intracellular delivery through other mechanisms. For example, changes in pH can alter the protonation status of ionizable functional groups on small molecules, affecting their interaction with the plasma membrane and transporters (23, 24). Furthermore, changes in pH will also affect broad cellular functions, including metabolism, vesicle trafficking, and endocytosis pathways (25). Since intracellular molecule delivery depends on all of these processes, pH levels may influence drug uptake by numerous mechanisms.

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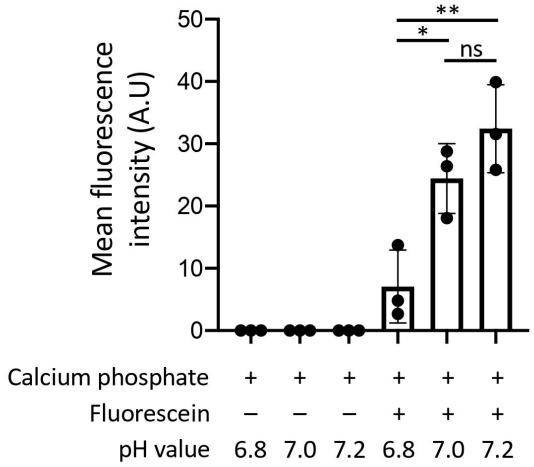


**Figure 2: Fluorescent intensities were higher in cells cultured in calcium phosphate/fluorescein-containing medium at higher pH.** Mean fluorescent signal intensity (wavelength 515 to 555 nm) of cells cultured in medium containing 0.1 M calcium phosphate with or with 100 nM fluorescein at pH levels of 6.8, 7.0, and 7.2. Results are expressed as mean ± standard deviation from three replicates, with three images per replicate analyzed (n = 3). (t-test with Bonferroni correction, *p < 0.05; **p < 0.025).

Although we cannot alter pH values within the intestines of our body to enhance drug absorption, we hope that our study will encourage consideration of body pH values as a factor in drug administration. Circadian rhythms, natural biological cycles that follow a roughly 24-hour pattern, have a profound impact on the physiology of the human body, including the gastrointestinal system (26). While gastric acid secretion varies between individuals, it commonly peaks between 10 pm to 2 am, implying that the efficiency of drug absorption during this period may differ from other times (27). In addition, pH fluctuations are also associated with various patient-specific factors, such as dietary habits, digestive activity, and hormone production (28). For example, the pH of the stomach decreases to 1.5–3.5 when gastric acid is actively produced but increases upon fasting or after meals (29). In the small intestine, pH levels fluctuate slightly depending on food consumption and time of day. The pH becomes more acidic upon consuming certain foods or drinks and more alkaline after meals due to the secretion of bicarbonate to neutralize gastric acid entering the duodenum (30). In addition to these normal physiological states, several pathological conditions, including kidney disease, diabetes, chronic vomiting or diarrhea, and cancer, can also affect pH levels (31-33). Therefore, while our study does not provide specific guidance on adjusting drug doses or formulas to improve absorption under various physiological or pathological conditions, we hope that our findings demonstrate the need to consider pH values in future drug manufacturing.

Regarding our methodology, several limitations in our study may have affected our results. For example, while calcium phosphate is typically mixed with therapeutic ingredients as an excipient or binder in drug tablets or capsules, we used a medium containing calcium phosphate and fluorescein to replace the original medium for cell culture, which can result

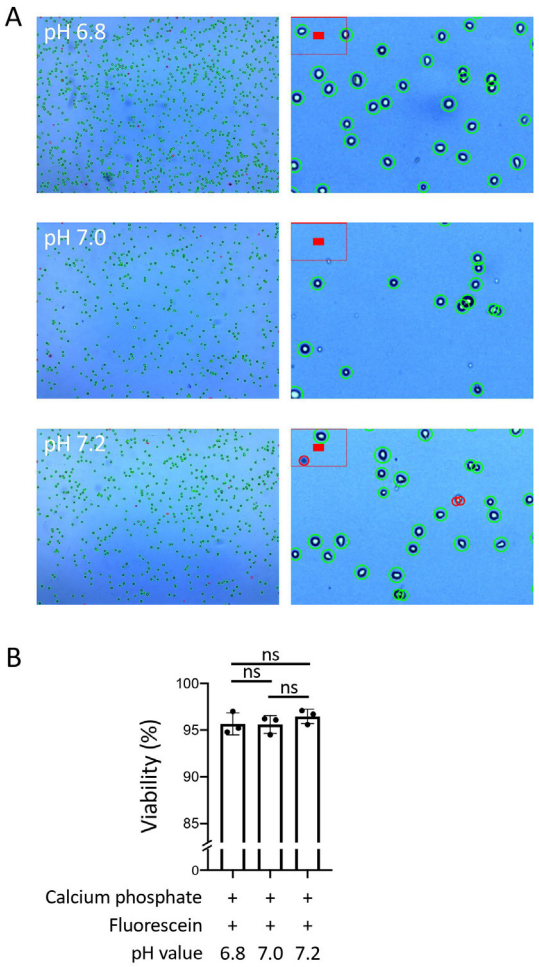


**Figure 3: Incubation of cells with calcium phosphate and fluorescein did not appreciably affect cell viability.** (A) Cells cultured in medium containing 0.1 M calcium phosphate and 100 nM fluorescein at pH levels of 6.8, 7.0, and 7.2 were trypsinized, stained with trypan blue, and subjected to cell counting and viability analysis using LUNA-II™ automated cell counter. The left panels show the full views captured by the cell counter. The right panels show enlarged views. Each individual cell was automatically identified and color-coded by the LUNA-II™ system. Green circles indicate live cells, whereas red circles indicate dead cells stained with trypan blue. (B) The mean viability of cells cultured in medium containing calcium phosphate and fluorescein at pH levels of 6.8, 7.0, and 7.2 was assessed by the LUNA-II™ automated cell counter. The results are expressed as mean ± standard deviation (n = 3). (t-test with Bonferroni correction, ns, not statistically significant).

in different pharmacodynamics (34). In addition, although the molecular weight of fluorescein falls within the range of the average molecular weights of small-molecule drugs, fluorescein may not exhibit the same uptake kinetics as other drugs or medications.

Future experiments could explore additional methods to modify the chemical properties of calcium phosphate or explore alternative substances for intracellular delivery. Beyond adjusting the pH of calcium phosphate, the introduction of different ions or modification of calcium phosphate could also influence its chemical properties. Moreover, investigating the efficacy of other substances for intracellular delivery could

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provide valuable insight into which drugs are best suited to a specific type of binder or filler. Examples of substances worth investigating include polyethyleneimine and magnesium phosphate, which have properties similar to calcium phosphate (35). Such research efforts could significantly advance biomedical delivery measures, potentially leading to the discovery of more effective alternatives to current delivery methods, which would in turn enhance drug efficacy.

In conclusion, our study highlights the relationship between pH levels and intracellular molecule delivery. Further research into alternative delivery substances and optimization methods could lead to improved biomedical applications and improved treatments for diverse medical conditions.

## MATERIALS AND METHODS

### Cell culture and fluorescein intracellular delivery at different pH levels

Caco-2 cells were maintained in culture in Dulbecco's Modified Eagle Medium (DMEM, Cat. No.11965092, ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS, No. 10082147, ThermoFisher Scientific), 2 mM L-glutamine (No. 25030081, ThermoFisher Scientific), 1X non-essential amino acids (No. 11140050, ThermoFisher Scientific), and 100 µg/mL penicillin-streptomycin (No. 15070063, ThermoFisher Scientific).

The medium containing calcium phosphate at pH 6.8, 7.0, and 7.2 was prepared as follows. First, 0.1 M calcium phosphate solution was prepared by mixing 3.1 g of solid calcium phosphate with 100 mL of Minimum Essential Medium (MEM, Cat. No.12492013, ThermoFisher Scientific). The pH was confirmed at 7.0 with a pH meter. Then, 0.057 mL of liquid acetic acid was added to the neutral calcium phosphate solution to a final pH of 6.8. Lastly, 0.002 mL of liquid ammonia was dissolved in the neutral calcium phosphate solution to a final pH of 7.2.

For cell culture, 1x105 Caco-2 cells were added to 1 mL DMEM in each well of sterilized 6-well plates and evenly dispersed. The 6-well plates were placed in an incubator (37°C, 5% $CO_2$, and atmospheric $O_2$) for 24 hours. To examine the effects of different pH levels on calcium phosphate-based drug delivery, a total of 2.0 mL of calcium phosphate solution prepared in MEM at pH levels of 6.8, 7.0, and 7.2 were transferred into three 10-mL test tubes. Fluorescein was then added to each tube to achieve a final concentration of 100 nM. The solutions were gently stirred at 25°C for 5 minutes to ensure thorough mixing. Afterwards, 1 mL of each fluorescein-containing medium was used to replace the original medium in each well of the 6-well plates for 6 hours. For the control samples, two sets of media were prepared. The first set included MEM with 0.1 M calcium phosphate at pH levels of 6.8, 7.0, and 7.2, but without fluorescein. The second set contained MEM with fluorescein at 100 nM, but without calcium phosphate. After 6 hours, the medium for the experimental and control groups was replaced with fresh DMEM medium, and the cells in the 6-well plates were imaged directly under fluorescence microscopes. Three microscopy images were captured for each replicate.

### Fluorescence signal analysis

Bright-field and fluorescence images were captured using a Leica DMi8 inverted microscope at 100X magnification with excitation at 488 nm and emission at 515 to 555 nm. Three replicates were prepared for each experimental condition, and three microscopy images were captured for each replicate. These images were analyzed with ImageJ software (version 1.54) to quantitatively assess fluorescein uptake. First, the images were converted to 8-bit format to enhance contrast and facilitate quantification. Next, we selected the entire image to estimate the mean gray value, which represents the fluorescence intensity across all imaged cells. We also counted the cell number in each image by converting bright-field images to 8-bit grayscale to distinguish cells. The images were then converted to binary, and the Analyze Particles function was used to quantify the number of cells.

### Cell viability analysis

To evaluate cell viability, cells cultured in medium containing calcium phosphate and fluorescein for 6 hours were trypsinized for the preparation of cell suspension. Next, 10 µL of the cell suspension was mixed with 10 µL of trypan blue dye. A 10 µL aliquot of the stained cell suspension was loaded onto a counting slide and inserted into a LUNA-II™ automated cell counter for cell counting and viability assessment.

### Statistics analysis

Statistical analysis was performed with GraphPad Prism 8. Statistical significance was assessed using a t-test with Bonferroni correction with an alpha of 0.05.

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